Targeting Cell Motility and Dissemination in Medulloblastoma

Dissertation

zur

Erlangung der naturwissenschaftlichen Doktorwürde
(Dr. sc. nat.)

vorgelegt der

Mathematisch-naturwissenschaftlichen Fakultät

der

Universität Zürich

von

Karthiga Santhana Kumar

von

Indien

Promotionskommission

PD Dr. Martin Baumgartner

PD Dr. Jean-Pierre Bourquin

Prof. Dr. Lucas Pelkmans

Prof. Dr. Gisbert Schneider

Prof. Dr. Britta Engelhardt

Zürich, 2017

TARGETING CELL MOTILITY AND DISSEMINATION IN MEDULLOBLASTOMA

A DOCTORAL THESIS

Submitted by

Karthiga Santhana Kumar

Thesis committee

PD Dr. Martin Baumgartner

PD Dr. Jean-Pierre Bourquin

Prof. Dr. Lucas Pelkmans

Prof. Dr. Gisbert Schneider

Prof. Dr. Britta Engelhardt

University of Zurich[UZH]

ETH Eidgenössische Technische Hochschule Zürich Swiss Federal Institute of Technology Zurich

cancer network zurich

cancer biology phd program

life science zurich graduate school

KINDERSPITAL ZÜRICH Universitäts-Kinderkliniken · Eleonorenstiftung

FNSNF

FONDS NATIONAL SUISSE
SCHWEIZERISCHER NATIONALFONDS
FONDO NAZIONALE SVIZZERO
SWISS NATIONAL SCIENCE FOUNDATION

SASSELLA FOUNDATION

University of Zurich, University Children's Hospital, Neuro-Oncology Group
Experimental Infectious Disease and Cancer research, August Forel Strasse 1, CH-8008 Zürich

Zusammenfassung

Medulloblastoma, (MB) ist der häufigste, maligne Gehirntumor im Kindes- und Jugendalter. Ein aggressives klinische Verhalten, welches durch die leptomenigeale Metastasierung (LMM) des MB verursacht wird, charakterisiert diesen vor allem im Kleinhirn auftretenden Tumor. LMM macht, zusätzlich zur Standardtherapie wie lokale Bestrahlung und Chemotherapie, die kraniospinale Radiotherapie notwendig. Leider verursachen diese sehr aggressiven und unspezifischen Behandlungen schwerwiegenden Spätfolgen, zu denen unter anderem auch endokrine Dysfunktionen und kognitive Beeinträchtigungen gehören, welche die Lebensqualität vieler Langzeitüberlebenden erheblich beeinträchtigen. Eine wichtige die LMM auslösende Ursache ist die deregulierte Kontrolle der Motilität der MB Zellen. Die spezifische Blockierung der unkontrollierten Zellmotilität könnte daher eine effiziente, weniger toxische anti-metastatische Therapie sein, indem sie die lokale Invasion reduziert, die weitere Metastasierung verhindert und einer generellen Entwicklung in Richtung Metastasierung entgegenwirkt. Um die metastatische Verbreitung des MB zu verhindern, sind daher neue Therapien notwendig, welche die Tumorzellmotilität gezielt blockieren.

Fundierte Kenntnisse der Mechanismen, welche die LMM des MB fördern sind notwendig, um neue anti-metastatische Therapien zu entwickeln. Das Ziel dieser Therapien wäre es die Überlebenswahrscheinlichkeit der Patientinnen und Patienten zu vergrössern und die therapieassoziierten Nebenwirkungen zu verringern. Die molekularen Mechanismen, welche die LMM steuern und regulieren sind nach wie vor unbekannt. Im Allgemeinen können sowohl intrinsische (Kinasen, die Regulation des Zellskeletts) als auch extrinsische (von der Tumorumgebung stammende) Faktoren die Metastasierung beeinflussen. Zu Beginn dieser Arbeit waren keine etablierten *in vitro* Methoden verfügbar, welche die potentielle Beteiligung von einer grossen Anzahl solcher möglichen Faktoren in der Regulation der Tumorzellverbreitung systematisch untersuchen liessen. Daher haben wir als ersten Schritt hin zu einem besseren Verständnis der MB Metastasierung eine Plattform zur qualitativen und quantitativen Analyse der Tumorzellmotilität in 2D und 3D Umgebungen entwickelt. Dieses Hochdurchflussverfahren, welches wir automatisierter Zelldisseminationszähler (aCDc) nannten, besteht aus zell-basierten Migrations- und Invasionsassays, einem Mikroskop zur Bildaufnahme sowie Softwarelösungen zur Quantifizierung der aufgenommenen Bilder.

aCDc erlaubte uns sowohl Parameter der Tumorumgebung als auch intrinsische Faktoren zu untersuchen, sowie effizient möglich Therapiestrategien auszutesten, welche die Tumorzellausbreitung verhindern könnten. Diese Studien ermöglichten die Identifizierung des Wachstumsfaktors HGF und der Kinase MAP4K4 als Schlüsselelemente der Disseminationsregulation. Wir zeigten, dass HGF in MB Zellen via die Aktivierung seines Rezeptors c-Met die Zellausbreitung im 2D sowie 3D Raum fördert. Wir konnten zusätzlich zeigen, dass die MAP4K4, welche die Dynamik des F-Aktin Zellskeletts in migrations- und invasionsrelevanten zellulären Fortsätzen kontrolliert, im Signalweg unterhalb des c-Met Rezeptors aktiv ist. Die Identifizierung dieses neuen, wachstumsfaktorinduzierten Signalwegs deute darauf hin, dass Wachstumsfaktoren und Zytokine aus der Tumorumgebung auch die Zellverbreitung des MB fördern könnten. Um diese Möglichkeit zu testen, untersuchten wir die Auswirkung verschiedener auch in der MB Tumorumgebung vorkommenden Wachstumsfaktoren und Zytokine auf die MB Tumorzellausbreitung.

Detaillierte Untersuchungen mittels aCDc demaskierten die Wachstumsfaktoren bFGF und EGF, zusätzlich zu HGF, als die wirksamsten Vermittler der MB Tumorzellausbreitung. Unter Benutzung von Zellen isoliert aus Xenografttumoren sowie von Kleinhirnschnittkulturen, identifizierte diese Studie bFGF als zentrale Komponente der Kontrolle von Tumorzellfunktionen durch Umgebungsfaktoren. Gestützt auf diese Erkenntnis, definierten wir die essentiellen Effektoren des bFGF Signalwegs, deren Blockade die bFGF-induzierte Tumorzellausbreitung stoppen könnte. Darüber hinaus untersuchten wir auch zusätzliche Signalwege auf ihren regulatorischen Einfluss bezüglich der bFGF-induzierten Signalübertragung. Wir wiesen nach, dass das Adaptermolekül FRS2 für die bFGF-induzierte Zellausbreitung notwendig ist. Ausserdem entdeckten wir, dass das Zytokin TGF-β die pro-metastatische Aktivität von FRS2 reprimiert, indem es FRS2 inaktiviert und in den Tumorzellen über die Aktivierung der Rho-Kinase ROCK eine morphologische Anpassung zu einem kontrahierten, nicht-motilen Phänotyp bewirkt. Wir wiesen nach, dass diese negative Regulation durch TGF-β von der Konzentration des vorhandenen bFGFs abhängt und dass der resultierende antagonistische Signaleffekt unabhängig der Konzentrationen von bFGF und TGF-β auf der Stufe von FRS2 konvergiert. Dies macht FRS2 zu einem sehr attraktiven Ziel für einen möglichen anti-metastatischen Therapieansatz für das MB. Um den antagonistischen Signaleffekt von bFGF und TGF-β therapeutisch auszunutzen, haben wir FRS2 als mögliches Therapieziel im MB validiert und die Schlüsselrolle von FRS2 auf molekulare Ebene für die Kontrolle der Kleinhirngewebeinvasion im MB nachgewiesen.

Dieses Projekt hat daher nicht nur ein neues antimetastatisches Zielmolekül im MB identifiziert, sondern auch einen einzigartigen antagonistischen Signaleffekt entdeckt, welcher die metastatischen Eigenschaften von MB Zellen kontrolliert. Damit ermöglichte diese Studie bedeutende Einblicke in zentrale Mechanismen der intrinsischen und extrinsischen Kontrolle der Tumorzellausbreitung, welche nicht nur für das MB sondern auch für andere solide Tumore von Bedeutung sein werden.

Summary

Medulloblastoma (MB) is the most common malignant pediatric brain tumor. MB is characterized by an aggressive clinical behavior, which is caused by leptomeningeal dissemination (LMD). LMD necessities aggressive treatment regimens like craniospinal radiotherapy, in addition to standard postoperative therapies such as local radiotherapy and chemotherapy. Unfortunately, such intensive, non-specific therapies causes severe late treatment-related complications including endocrine and growth dysfunctions and cognitive impairments, thereby decreasing the quality of life for many long-term survivors. An important cause of LMD is the deregulated motile behavior of MB cells. The specific blockade of this de-regulated tumor cell motility may represent an efficient, less toxic, anti-metastatic strategy for MB through the reduction of local invasion of MB cells, the prevention of further dissemination and the repression of the evolution towards increasingly metastatic phenotypes. Novel therapies targeting the dissemination process are thus urgently needed.

New insights into the mechanisms promoting MB leptomeningeal dissemination are required to develop novel anti-metastatic therapies, which in turn can increase patient survival and reduce treatment-related side effects. However, the molecular mechanisms that tune the LMD remain enigmatic. Generally, both cell intrinsic (kinases, actin dynamics) and extrinsic factors (tumour microenvironment) can influence metastasis. There were no established systematic *in vitro* approaches available to study the potential contribution of a vast number of factors that could regulate MB cell dissemination. Therefore, as first step towards understanding and targeting MB cell dissemination, we developed an assay platform to measure cancer cell dissemination parameters qualitatively and quantitatively in 2D and 3D environments in high-throughput. This platform, which we named automated Cell Dissemination counter (aCDc), consists of cell-based migration / invasion assays coupled imaging devices for acquisition and software solutions for the quantification of the imaging data.

aCDc enabled us to place emphasis on both the tumour microenvironment parameters and intrinsic factors and to explore strategies to target MB cell motility effectively. It led to the identification of the growth factor HGF and MAP4K4 as key players of MB cell dissemination. We demonstrated that HGF in MB cells via the activation of its receptor c-Met promotes MB cell dissemination in 2D and 3D environments. Furthermore, we showed that MAP4K4, which controls F-actin cytoskeleton dynamics in cellular protrusions necessary for motility and invasiveness, acts downstream of the HGF signaling pathway in MB. Identification of this novel growth factor-induced, MAP4K4-dependent signaling circuit hinted that other growth factors and cytokines released from the microenvironment could also promote MB cell dissemination. To explore this possibility, we studied the impact of various growth factors and cytokines in the tumour microenvironment of MB on MB cell dissemination.

Detailed analysis using aCDc revealed bFGF and EGF as strongest promoters of MB cell dissemination, in addition to HGF. Using cells derived from patient derived xenografts and *ex vivo* slice culture models, this study identified bFGF as a central component of micro-environmental control of pro-metastatic tumour cell functions in MB. By virtue of this, we explored the various downstream effectors of FGFR signaling, whose blockade may inhibit bFGF-induced dissemination, and also evaluated other signaling cascades that may modulate FGFR signaling. We found that the adapter

protein FRS2 mediates bFGF-dependent migration and invasion. Furthermore, we discovered that TGF-β inhibits the pro-metastatic functions of FRS2 by repressing its activity and by causing a morphological switch to a non-migratory, contractile phenotype via Rho-ROCK activation. We also uncovered the context-dependent signaling of TGF-β in the presence of bFGF gradients in MB. Irrespective of the concentration of bFGF and the presence or absence of TGF-β, this antagonistic crosstalk between bFGF and TGF-β signaling converges at the level of FRS2, which renders it an attractive target for an anti-dissemination therapy approach in MB. Towards exploiting this crosstalk therapeutically to target MB cell dissemination, we validated FRS2 as a potential anti-metastatic therapy target in MB and demonstrated the key role of FRS2 at the molecular level for the control of cerebellum tissue infiltration in MB.

This project identified a novel target to specifically inhibit MB cell dissemination and has unraveled a unique crosstalk dependent regulation of metastatic capabilities of MB cells. Thus, this study provides key insights into intrinsic and extrinsic control of tumour dissemination, which are not only of high significance for the MB but also for other solid tumours.

Table of Contents

1. Introduction

- Pediatric cancers
- Childhood brain cancers
- Medulloblastoma
- Tumour microenvironment
- Cell motility and cancer
- Cell motility models
- MAP4K4
- Fibroblast growth factor receptor signaling
- Transforming growth factor receptor signaling

Targeting cell motility and dissemination in medulloblastoma

1. Introduction:

1.1 Pediatric cancers

1.1.1 Cancer in childhood and adolescents – epidemiology, incidence, classification and survival

Cancer is a global and a heterogeneous disease with many intricate aspects. A total of 1,685,210 new cancer cases and 595,690 cancer deaths have been reported in the United States in 2016 [1]. In Switzerland, almost 35,000 people are diagnosed with cancer and more than 16,000 cases results in death per year [2].

Epidemiology and Incidence: Around 1% of all newly diagnosed cases occur in children and adolescents in developed countries [3]. 5,776 children (age 0-14 years) [4] were diagnosed with cancer from 1985 to 2014 in Switzerland [5] and 37 pediatric cancer-related deaths are reported every year [2] (Figure 1A, registered by the Federal Statistical Office (FSO), the National Institute for Cancer Epidemiology and Registration (NICER) and the Swiss Childhood Cancer Registry (SCCR)). Despite a subsided number of cases and cure rates for childhood cancers being impressive relative to those for adult malignancies, cancer remains to be the leading cause of death by disease among children over 1 year of age in developed countries [6]. Overall cancer incidence trends recorded for the last 5 years (from 2009-2012) for all cancers remained stable in women, declined by 3.1% in men, while it is increased by 0.6% in children [1].

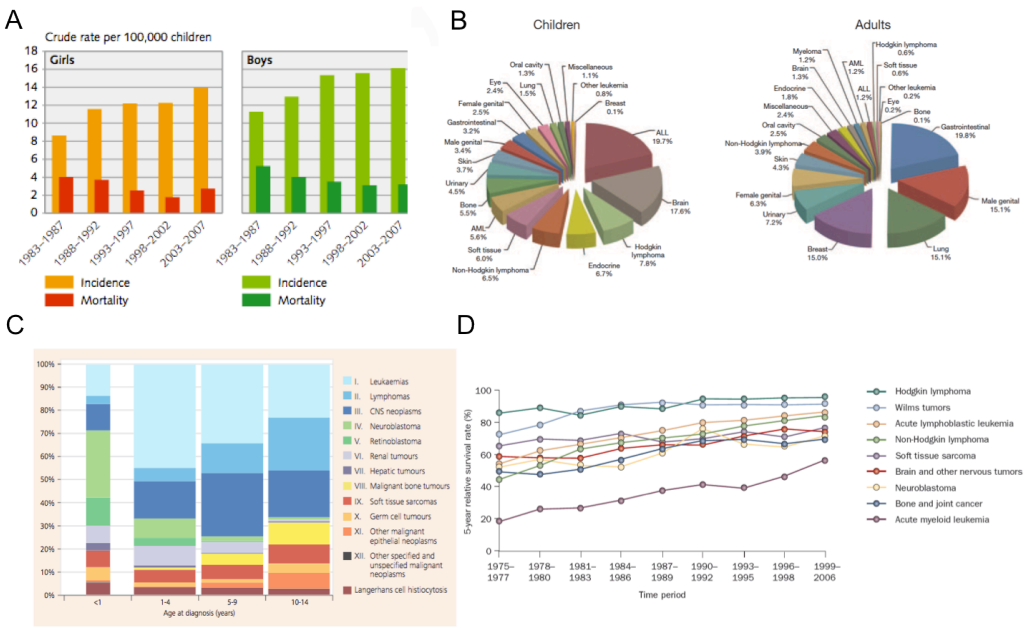


Figure 1: The distribution, incidence and survival rates of pediatric cancers. (A) The incidence of childhood cancers from early 1990s till 2007 for both genders in Switzerland (Adapted from [7]) **(B)** The differences in

the spectrum of tumours between children (left) and adults (right) as reported by SEER data 2012 (Adapted from [8]). **(C)** Childhood cancer distribution within the age groups in Switzerland. (Adapted from [2]). **(D)** Comparison of 5-year survival rates for pediatric cancers at reported by SEER data 2014. (Adapted from [9])

The spectrum of cancer varies largely between children, adolescents and adults (Figure 1B). While carcinomas like breast, lung, prostrate and colorectal cancers are predominant in adults; leukemia, brain and solid tumours arise most commonly in children [6]. Leukemia presents with specific subtypes and with high incidence rates in children but it can also occur in all age groups. Medulloblastoma and a variety of pediatric sarcoma subtypes like rhabdomyosarcoma, Ewing sarcoma, osteosarcoma and Wilms tumour can arise in adults, but are extremely rare [10].

Classification: The third and the most updated version of International Classification of Childhood Cancer (ICCC-3) has coded childhood cancers into 12 main groups, which are further classified into 47 subgroups [11]. The 12 main sub-groups of pediatric cancers are

I. Leukemia, myeloproliferative diseases, and myelodysplastic diseases
II. Lymphomas and reticuloendothelial neoplasms
III. Central nervous system and miscellaneous intracranial and intraspinal neoplasms
IV. Neuroblastoma and other peripheral nervous cell tumours
V. Retinoblastoma
VI. Renal tumours
VII. Hepatic tumours
VIII. Malignant bone tumours
IX. Soft tissue and other extraosseous sacromas
X. Germ cell tumours, trophoblastic tumours and neoplasm of gonads
XI. Other malignant epithelial neoplasms and malignant melanomas
XII. Other and unspecified malignant neoplasms

The most common malignancies that occur in the age range from 1-14 years are acute lymphoblastic leukemia (26%), tumours of the central nervous system (21%), neuroblastoma (7%) and non-Hodgkin lymphoma (6%) [10]. The incidence trends shifts towards Hodgkin lymphomas (15%) in adolescents (15-19 years), followed by thyroid tumours (11%) and the tumours of brain and the central nervous system (10%). A variety of cancers prevalent in adolescent young adults have a worse outcome than the same cancers in younger or older patients [12].

Survival: Pediatric cancers relish a striking survival rate of around 80%, which is much higher as compared to adults. The 5-year relative survival rate for all cancers combined has dramatically increased from 1999-2006 as analyzed by SEER (Surveillance, Epidemiology and End results) program of the National Cancer Institute (seer.cancer.gov). Among children younger than 1 year of age, 5-year survival rates for all cancers combined reached 78.2% relative to 60% observed in late 1970s and 1980s. 5-year survival increased from approximately 60% in 1975-1978 to 80.6% in 1999-2002 in children 1-14 years of age. Among children 15-19 years of age, 5-year survival rates increased from 67.6% in 1975-1978

to 79.4% in 1999-2002 [13]. However, the outcome is very heterogeneous within the pediatric tumour subgroups (Figure 1C, 1D) and it highly depends on the disease state, abnormalities and the age of the patient. Best survival rates with ≥ 85% have been reported for Hodgkin lymphoma, Wilms tumour and acute lymphoblastic leukemia. Even though, the survival rates are improving for leukemia and lymphoma, the rates for solid tumours have reached a plateau since the last decade. Little or no progress has been made for children with malignant glioma, brain and other nervous tumours and sarcomas highlighting the need for efficient targeted therapies [14].

1.1.2 Childhood cancer – treatment and long-term follow-up

Treatment: Treatment of pediatric cancer patients includes cytotoxic chemotherapy, radiotherapy and surgery in the case of solid tumours. The exact treatment protocols for childhood cancers are dependent on the tumour type and sub-group and can slightly vary between Europe and US [14]. Although, roughly 250 children globally lose their lives to cancer, 80% of all childhood cancers are potentially curable with present treatment strategies [15]. The good survival rates for most pediatric cancers result from continuous efforts of prospective clinical trials, improved risk assessment and supportive care [16]. Treatment related toxicities and adverse side effects are the most challenging aspects of pediatric cancer therapy.

Long-term follow-up: Children who survived a pediatric cancer may experience long-term treatment related side effects many years post-diagnosis. Childhood cancer survivors are at risk of developing secondary malignant neoplasms. In general, the likelihood of developing a second primary cancer appears to be approximately 10 times higher than the general population of the same age [17]. A 30-year follow-up study (1970-1986) of the Childhood Cancer Survivor Study (CCSS) cohort showed a 9.3% increase in the incidence for secondary malignancies [18]. The secondary neoplasms are strongly influenced by the original diagnosis and therapy and the risk of secondary cancer is increased by the use of therapeutic radiation and intensive chemotherapy. Perceptible investigations with childhood cancer survivors have shown excessive mortality rates within 5 years of treatment [19]. A larger cohort study consisting of 34,033 childhood cancer survivors, with 21 years median follow-up time revealed that there were 3,958 deaths with 51% due to recurrence or progression of the primary tumour, 8% to external causes and 41% to health related causes. Among the health related causes, 41% was due to subsequent neoplasms, 15% from cardiac causes, 8% from pulmonary causes and 31% from other causes [20] [4]. The overall cumulative mortality rate at 30 years after diagnosis was 18.1%, implying long-term morbidity as a serious issue for later life [18].

Clinically focused follow-up and monitoring of childhood cancer survivors are extremely necessary for secondary malignancies and health issues associated with cancer treatment early in life. It is believed that improved treatment protocols and new specific targeted therapies will decrease the risk of secondary neoplasms and treatment related health issues in the future.

1.1.3 Genomic landscape of adult and pediatric tumours: A comparison

Cancer genomes are largely characterized by genomic instability, accumulation of gene mutations and epigenetic changes vitalizing tumour initiation, progression and maintenance [21]. The tumour cells ensure their own survival and spreading throughout the human body by manipulating the most crucial pathways as follows:

1. Sustaining proliferative signaling
2. Evading growth suppressors
3. Avoiding immune destruction
4. Enabling replicative immortality
5. Tumour-promoting inflammation
6. Activating invasion and metastasis
7. Inducing angiogenesis
8. Genome instability and mutation
9. Resisting cell death
10. Deregulating cellular energetics

The various ways by which a cancer cell can regulate the aforementioned pathways are summarized in the hallmarks of cancer [22]. Although there are diversified theories and models for cancer development, in most adult cancers, tumour formation is based on two constitutive processes: a) the continuous acquisition of heritable genetic variation in individual cells by more or less random mutations, b) natural selection of the resultant phenotypic diversity. It was first shown in colorectal cancer that a sequential series of mutations resulted in activated oncogenes and / or loss of function of critical tumour suppressor genes that lead the progression of benign lesions to malignant phenotype [23] [24]. Since then, various efforts have been made to characterize the human cancer genome. It has been reported that at least 350 (1.6%) of the ≈ 22,000 protein-coding genes in the human genome show mutations in cancer [25]. The identification of numerous different mutational patterns within the same tumour type sub-type implied that not all mutations cause cancer. The so-called 'Driver' mutations confer a growth advantage to cancer cells whereas 'passenger' mutations do not. Driver mutations are found with a high incidence in distinct tumour entities, but are more infrequent in others, suggesting a heterogeneous mutational landscape for all tumour types in adult cancers [23]. Mutations in tumours such as gastric, lung, colorectal cancer or melanoma are highly subjective based on the exposure to exogenous mutagens. They exhibit a high prevalence for accumulation of somatic mutations due to their epithelial origin and high cellular turn over rate. Several melanoma and lung cancer subtypes display more than 1000 mutations per base (Mb) DNA [26].

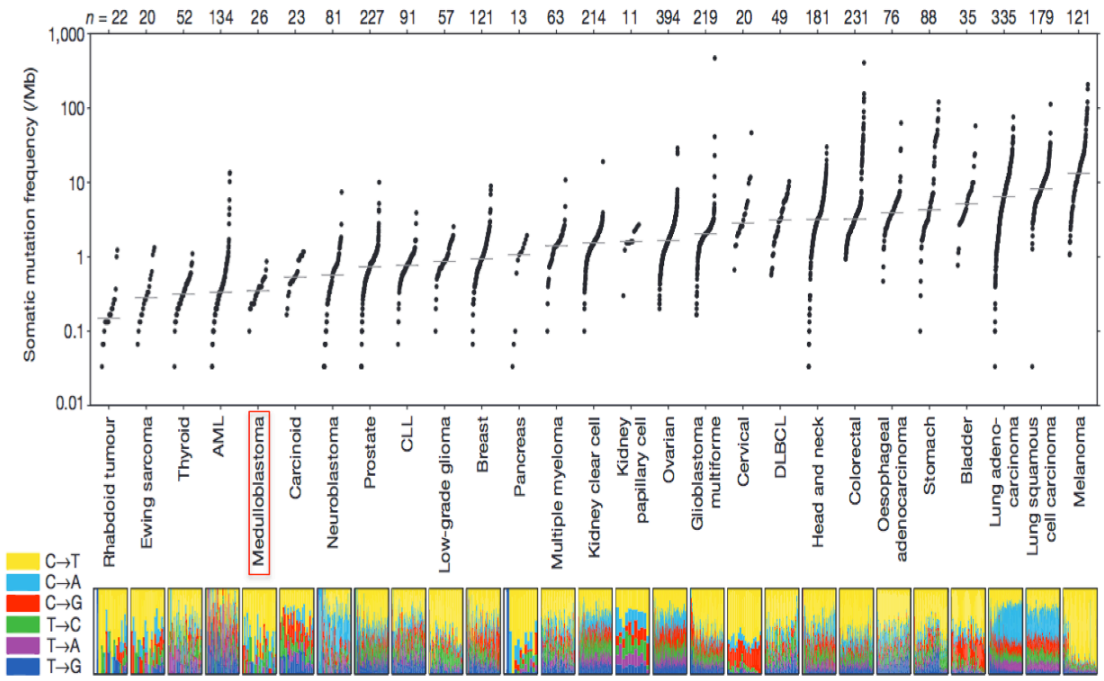


Figure 2: The genomic landscape of tumors: Somatic mutation frequencies are shown for selected cancers with the lowest to highest frequencies from the left to the right panel. The dots represent the mutation frequency in exomes from tumor versus normal pairs. Mutation frequencies highly vary between different caners, pediatric cancers including medulloblastoma cluster on the left site (Adapted from [25]). (Mb= mega base)

In contrast, pediatric cancers are characterized by a low frequency of mutations with usually less than one mutation / Mb DNA. Medulloblastoma (MB) has one of the lowest mutation frequencies among all pediatric cancers with less than 0.1 mutation/Mb DNA (Figure 2) [27]. A key finding from the Pediatric Cancer genome project is the extent to which the molecular characteristics of childhood cancers correlate with their tissue (cell) of origin [6]. As seen with most adult cancers, mutations in childhood cancers are not randomly distributed across the disease. Instead, distinctive oncogenic insults are associated with specific susceptible cell types and time windows of vulnerability. The presence of H3.3 and H3.1 K27 mutations almost exclusively in pediatric high-grade gliomas [28], the loss of *SMARCB1* in rhabdoid tumours [29], specific translocations in specific types of leukemia [30] and the presence of specific fusion genes in pediatric sarcomas [31] highlight the existence of explicit mutations in particular sub-types of pediatric cancers. Apart from the specific translocations and fusion proteins, copy number alterations of genes involved in normal development of the tissue of origin and alterations in the epigenomic regulation contribute to the pediatric cancer pathogenesis [32]. Taken together, structural variations and translocations govern many childhood cancers like leukemia and sarcoma while fusion proteins and overexpression of oncogenes play a central role in pediatric cancer development. However, there are childhood cancers where recurring structural variations or fusion genes have not been found. For example, translocations confined to the first intron of *TP53* in osteosarcoma [33] and juxtaposition of GFI1 or GFI1B coding sequences proximal to active enhancer elements leading to transcriptional activation (so-called 'enhancer hijacking') in medulloblastoma [34] can also have oncogenic effects. The oncogenic mechanisms for certain cancers like neuroblastoma [35] are still unclear and remain to be elucidated.

1.2 Childhood brain tumours

1.2.1 Pediatric brain tumours – epidemiology, incidence, classification and survival

Pediatric central nervous system (CNS) neoplasms (including the brain and spinal cord) include a spectrum of both glial and non-glial tumours that differ significantly in location and biological behavior from those of adults. As a group, they make up 25% of all childhood malignancies and represent the most common solid tumour of childhood [36].

Epidemiology: Each year, the American Cancer Society estimates the numbers of new cancer cases that will occur and complies the most recent data on cancer incidence, mortality and survival. In 2016, the most recent SEER program report showed that among children and adolescents (aged birth-19 years), brain cancer has surpassed leukemia as the leading cause of cancer deaths because of the dramatic therapeutic advances against leukemia [13]. This calls for an accelerating progress against brain cancer therapeutics.

Incidence: The largest information data sets on childhood brain tumours are available through the Central Brain Tumour Registry of the USA (CBTRUS). According to the current CBTRUS report (updated Feb 2011), pediatric brain tumours (including all primary CNS tumours) have an annual incidence of 4.84 per 100,000 population in the 0-19 year age group [37]. Based on these data, approximately 4000 people under the age of 20 are expected to develop primary brain tumour in the USA each year. The prevalence of primary CNS tumours (0-19 years of age) is estimated at 35.4 per 100,000 population, implying that over 28,000 children are currently living with this diagnosis in the USA. The SEER data reports a slight difference in incidence rate by race and gender. Primary CNS tumours are more common in whites than blacks (5.02 vs 3.69 per 100,000) and males have a higher incidence rate (4.9 vs 4.8 per 100,000) as compared to females [37]. In Europe, Automated Childhood Cancer Information System (ACCIS) analyzed a total of 19,531 cases (0-14 years) and reported an overall age-standardized incidence rate of 29.9 per million [38]. There is a measurable rise in the diagnosis and early detection of brain tumours in children, which is associated with the advent of magnetic resonance imaging scanning in the developed world. There are only 2 (well defined) risk factors associated with the increased likelihood for the development of a primary CNS tumour in childhood: having a history of receiving significant doses of radiation to the CNS and / or having been born with inherent genetic predispositions to brain tumours [39].

Classification: The pediatric brain tumours are broadly classified into 2 classes as glial tumours and non-glial tumours based on the cell of origin of the tumours [39] [40]. Glial tissue is a scaffolding network and connective tissue array found throughout the CNS that plays different roles in different parts of the brain. Thus, glial origin tumours can be found in different parts of the brain and spinal cord. Non-glial tumours have an embryonal or glandular origin. Based on the above classification, glial tumours include astrocytic, oligogendrogial, oligoastrocytic and ependymal tumours whereas the non-glial tumours include embryonal tumours and choroid-plexus carcinomas. The main categories of childhood NS tumours are listed in Table 1

Table 1: Main categories of childhood CNS tumours (Adapted from [40])

Glial origin tumours

• Astrocytoma and other gliomas
  o Low grade
  o High grade

• Ependymoma
Non-glial origin tumours

• Embryonal tumours
  o Medulloblastoma
  o CNS Primitive neuo-ectodermal tumours (PNET)
  o Atypical Teratoid Rhabdoid Tumour

• Choroid plexus Tumours
  o Papiloma
  o Carcinoma

• Germ cell tumours
  o Germinoma
  o Non-germinomatous germ cell tumour

• Craniopharyngiomas

Survival: World Health Organization (WHO) tumour grade (I: low grade (well differentiated) – IV: high grade (Anaplastic)) is one component of a combination of criteria used to predict the response to therapy and disease outcome. CNS tumour patients with WHO grade II tumours typically survive more than 5 years and those with grade III tumuors survive 2-3 years. The prognosis of the patients with WHO grade IV tumours largely depends on the efficacy of the treatment regimens [41]. WHO tumour-grading system categorizes embryonal pediatric tumours like medulloblastoma and germinomas as grade IV, which can be rapidly fatal, if untreated (Table 2). With advances in the state-of-the-art radiation and chemotherapy, 5-year survival rates exceed 60 and 80%, respectively in pediatric CNS tumours [41] [42]. European analysis of the childhood central nervous system tumours (1978-1997) reported an overall 5-year survival of 64%, which varied between 72% in the North and 53% in the East. Among the various subgroups of pediatric CNS tumours analyzed, PNET had the poorest prognosis (49%) and astrocytoma the best (75%). Survival has improved by 29% since late 1970s [38].

Table 2: WHO classification (2007) of CNS tumours based on tumour grade. (Adapted from [41])

	I	II	III	IV
Astrocytic tumours				
Subependymal giant cell astrocytoma	•			
Pilocytic astrocytoma	•			
Pilomyxoid astrocytoma		•		
Diffuse astrocytoma		•		
Pleomorphic xanthoastrocytoma		•		
Anaplastic astrocytoma			•	
Glioblastoma				•
Giant cell glioblastoma				•
Gliosarcoma				•
Oligodendroglial tumours				
Oligodendroglioma		•		
Anaplastic oligodendroglioma			•	
Oligoastrocytic tumours				
Oligoastrocytoma		•		
Anaplastic oligoastrocytoma			•	
Ependymal tumours				
Subependymoma	•			
Myxopapillary ependymoma	•			
Ependymoma		•		
Anaplastic ependymoma			•	
Choroid plexus tumours				
Choroid plexus papilloma	•			
Atypical choroid plexus papilloma		•		
Choroid plexus carcinoma			•	
Other neuroepithelial tumours				
Angiocentric glioma	•			
Chordoid glioma of the third ventricle		•		
Neuronal and mixed neuronal-glial tumours				
Gangliocytoma	•			
Ganglioglioma	•			
Anaplastic ganglioglioma			•	
Desmoplastic infantile astrocytoma and ganglioglioma	•			
Dysembryoplastic neuroepithelial tumour	•			
Central neurocytoma		•		
Extraventricular neurocytoma		•		
Cerebellar liponeurocytoma		•		
Paraganglioma of the spinal cord	•			
Papillary glioneuronal tumour	•			
Rosette-forming glioneuronal tumour of the fourth ventricle	•			
Pineal tumours				
Pineocytoma	•			
Pineal parenchymal tumour of intermediate differentiation		•	•	
Pineoblastoma				•
Papillary tumour of the pineal region		•	•	
Embryonal tumours				
Medulloblastoma				•
CNS primitive neuroectodermal tumour (PNET)				•
Atypical teratoid / rhabdoid tumour				•
Tumours of the cranial and paraspinal nerves				
Schwannoma	•			
Neurofibroma	•			
Perineurioma	•	•	•	
Malignant peripheral nerve sheath tumour (MPNST)		•	•	•
Meningeal tumours				
Meningioma	•			
Atypical meningioma		•		
Anaplastic / malignant meningioma			•	
Haemangiopericytoma		•		
Anaplastic haemangiopericytoma			•	
Haemangioblastoma	•			
Tumours of the sellar region				
Craniopharyngioma	•			
Granular cell tumour of the neurohypophysis	•			
Pituicytoma	•			
Spindle cell oncocytoma of the adenohypophysis	•			

1.2.2 Pediatric brain tumours – treatment, current therapies and late effects

Treatment: The treatment and care of children with brain tumours are increasingly complex and require an array of disciplines and resources from within the health care systems. For any pediatric solid tumour, the basic principles of treatment rely on a combination of surgery, radiation and chemotherapy [39]. Surgery is needed to treat most pediatric brain tumours and it forms the central component of any brain tumour program [43]. Radiation is given in daily fractions in an outpatient setting, usually for a total course lasting for 6 or 7 weeks [44]. A different technology known as 'proton' radiation is being offered in a growing number of centers in the USA and Europe [45]. Although the total dose delivered to the

tumour remains the same with protons, there is ability to provide a more focused beam with sharper margins, thus reducing long-term side effects in young children [46, 47].

Chemotherapy was incorporated more recently in the treatment regimens of pediatric CNS tumours than surgery or radiation. Multiple challenges exist in delivering chemotherapy to the CNS, including crossing the blood-brain barrier [48]. Despite the hurdles, chemotherapy is being successfully used in the following 4 ways in the treatment of pediatric CNS tumours.

I. Low doses of chemotherapy are used over extended periods in an attempt to slow or halt the growth of low-grade tumours. For example, the weekly use of intravenous vincristine and carboplatinum in the management of unresectable low-grade gliomas [49].
II. High doses of chemotherapy are used as adjuvant (after) or neoadjuvant (before) treatment to enhance treatment with surgery and / or radiation [48]. The classic example is in medulloblastoma, where patients will have a better chance of survival if they receive chemotherapy after their surgery and radiation [50].
III. High doses of multi-agent chemotherapy are being used to treat infants and young children to prevent or delay the need for radiation therapy. Over the past 2 decades this treatment approach has been used with some success. However, it must be recognized that these treatment are acutely more toxic than the standard radiation techniques. For youngest patients, this has to be balanced against the devastating long-term effects of radiation [51].
IV. Chemotherapy can be used a radiation 'sensitizer', where patients will receive daily or weekly chemotherapy during radiation therapy, to increase its efficacy in some tumours [52].

Current therapies: To further enhance the survival rates and to recede the long-term adverse effects in pediatric CNS patients, ongoing clinical trials are testing investigational agents, combination chemotherapy and different radiation therapy regimens [53, 54]. The complete list of pediatric clinical trials for brain tumours can be found at the US National Institute of Health website: http://www.clinicaltrials.gov. The benefit of anti-angiogenesis therapy with antibodies against Vascular Endothelial Growth Factor (VEGF) in recurrent ependymomas [55], Erlotinib in Progressive Low-grade Glioma [56], Bevacizumab and Cediranib in refractory / recurrent medulloblastoma and PNETs [55, 57] are some of the highlights of current investigations.

Late effects: The late effects of brain tumours in childhood can manifest in a wide array of problems due to the tumour, surgery, radiation therapy and / or chemotherapy. Neuro-cognitive decline, memory difficulties, social skill deficits, secondary malignancies, neurologic deficits, seizures, growth deficiencies and endocrinopathies are few of the many late effects aspects requiring long-term care in pediatric patients [46, 58]. Late effects related to tumours occur in tumours that develop in an unresectable location (e.g. brain stem, thalamus) and can lead to significant motor and neurological dysfunction [59]. A late effect associated with surgery is an entity known as 'posterior fossa mutism syndrome' (also known as cerebellar mutism). Soon after the postoperative period, patients become unable

to speak or express themselves and exhibit pronounced mood dysregulation and hypotonia [60]. Late effects with regard to radiation therapy usually differ from patient to patient and the most common is an increased risk of ischemia and stroke [61]. With the progressive use of chemotherapy in treatment of pediatric brain tumours, the most harmful late effect of chemotherapy is formation of secondary malignancies. Alkylating agents, such as cyclophosphamide can also have a dose-dependent impact on fertility. Platinum-containing drugs are indispensible in the treatment of brain tumours, but come with an increased risk of sensorineural hearing loss and kidney damage. Anthracyclines (like doxorubucin) are less commonly used in pediatric brain tumours, but exposure to these medications is associated with long-term cardiotoxicity [62, 63].

The major effort today lies in improving the long-term quality of life, particularly in those children, who survive their disease but commonly suffer morbidity from treatment or the tumour itself.

1.3 Medulloblastoma

1.3.1 Origin, development and progression

Medulloblastoma (MB) is the most common malignant brain tumour of childhood. MB commonly occurs in the children as a midline posterior fossa mass arising from the cerebellar vermis, which appears as a hyper-dense, homogenous mass on (Computed tomography) CT scan (Figure 3A) [64]. This unique clinic-radiological pattern is considered 'typical', but significant number of MB cases does not follow the typical clinical patterns. Patients (mostly adults) can also present MB located laterally in the cerebellar hemisphere with / without calcification of the tumour mass [65, 66]. Very rarely, MB can also be presented in a very unusual location (i.e.) the cerebellopontine angle cistern (Figure 3B, 3C) [67, 68].

Origin: Since the medulloblastoma was first distinguished from other brain tumours in 1910, it has been considered to arise from neural precursor cells located in or near the cerebellum [69]. Although, the precise cells of origin of MB remains to be determined, perceptible evidence prove that this disease is intimately related to the development of the normal cerebellum. Microanatomically, the human cerebellum is composed of three distinct layers: molecular layer (also called external granular layer), Purkinje cell layer and the granular cell layer (inner granular layer). The layers of the cerebellum are well protected by the three defined meningeal layers (Figure 3D). Three differentiated cell types are found in MB: neurons, glia and muscle cells. Because of the presence of multiple differentiated cell types these tumours are aptly named after the postulated cerebellar stem cell, the 'medulloblast', that would give rise to the differentiated cells found in the tumours [70]. Despite various cells contributing to the development of MB, MB predominately originate from the granule-cell progenitors that are located in the external granular layer (EGL) of the cerebellum (Figure 3E). This is the germinal layer harboring actively proliferating progenitor cells (Granule Neuron Precursor cells (GNPCs)) originating from the rhombic lip during embryonic development [71]. A tightly regulated switch to the post-mitotic state correlates with

differentiation and inward migration of the granule neurons past the Purkinje cell layer to form the internal granule layer (IGL) [72]. An interesting property, of progenitor cells is their ability to undergo asymmetric division in which one daughter cell remains progenitor-like and the other then subsequently differentiates into a precursor cell type. This characteristic asymmetric division of the progenitor cells is susceptible to mutation and might serve as cells of origin in medulloblastoma and its variants [73].

Development: To avoid mutations in the distinct precursor cell populations that form the cerebellum require a series of controlled and coordinated cell signals to function. The bone morphogenic proteins (BMPs) Bmp6, Bmp7 and Gdf7 produced by cells of the dorsal midline provide signal that initiates the program of granule cell specification within cells of the rhombic lip [72, 74]. Following migration into the EGL, GNPCs proliferate in response to the mitogen sonic hedgehog (SHH), which is secreted by Purkinje cells [75, 76]. In addition, basic fibroblast growth factor (bFGF) signaling via extracellular signal-regulated kinase [76] and c-Jun N-terminal kinase (JNK) [77] potentially inhibits the proliferative response of GNPCs to SHH. Furthermore, Notch cell signaling pathway also orchestrates the proliferation and differentiation of GNPCs [78]. Mutations in the signaling pathways and de-regulation of the same, subvert normal cerebellar developmental programs and results in the formation of distinct types of medulloblastoma.

Progression: Metastatic spread along the leptomeninges (space between the pia mater and arachnoid mater of the meninges) has been reported for virtually all types of CNS tumours but it is most frequent in medulloblastoma. MB cells have an inherent propensity to disseminate via the leptomeningeal spaces of the brain and the spinal cord, thus leading to formation of secondary tumours (Figure 3F, 3G) [79]. Leptomeningeal disease (LMD) occurs in up to 32% of children with MB at diagnosis and it 100% positively correlates with worse prognosis which necessitates more aggressive therapy. The various therapy options for MB patients entirely depend on the presence or absence of LMD. Therefore, this requires a careful evaluation for evidence of LMD at the time of diagnosis and disease relapse [80]. The reason why some tumours disseminate while others do not remains speculative. Some studies hint that tumours that contained regions of glial, ependymal and / or neural differentiation were more likely to disseminate [81]. Other cell specific evidences like presence of specific microRNA (miRNA-182) also show a proclivity towards LMD [82]. However, the factors responsible for dissemination and the precise molecular mechanisms involved in LMD remains elusive. For decades, MB metastases have been assumed to be biologically similar to the primary tumour [83, 84]. A recent study with mouse and human MB show that metastases from an individual are extremely similar among them but are divergent from the matched primary tumour. Clonal genetic events in the metastases can be demonstrated in a restricted sub-clone of primary tumour, suggesting that only rare cells (a sub population) with the primary tumour have the ability to metastasize [85]. This bi-compartmental nature of metastatic MB has to be considered during development of therapeutic targets. Failure to do so may result in selection of therapeutic targets present in the primary tumour, which is more amenable to surgical control but not the metastases, which are the more frequent cause of death.

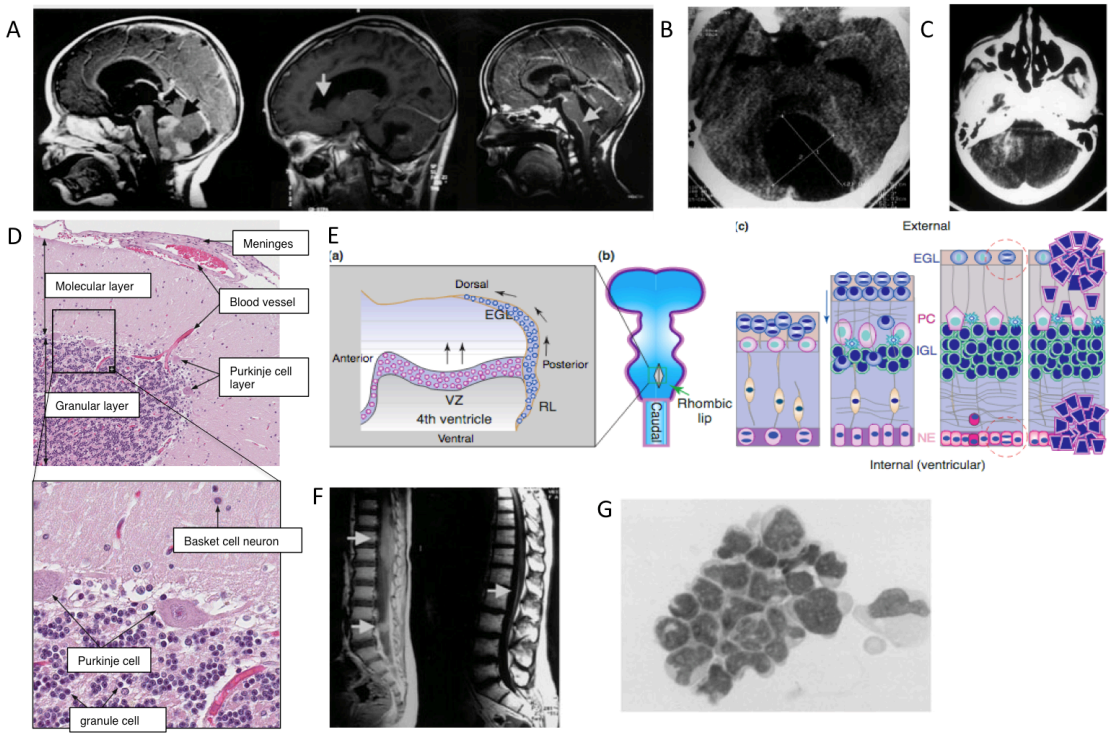


Figure 3: The origins and development of medulloblastoma: (A) Contrast enhanced computed tomography (CECT) showing MB in the cerebellum. (Adapted from [66]) (B, C) Uncommon presentation of MB (Adapted from [68]). **(B)** CECT showing a vermian medulloblastoma with a predominant cystic component. **(C)** CECT revealing a medulloblastoma in the cerebellopoentine angle (confirmed on biopsy). **(D)** Hematoxylin and eosin staining of a section of human cerebellum representing the normal histology and layers of cerebellum (Adapted from the 'The human protein atlas') **(E)** Schematic representation of the development of cerebellum: (a), (b) embryonal development of progenitor cells in the rhombic lip (RL) migrate dorsally to the external granular layer (EGL). (c) Postnatal development of cerebellum. The sites of origin of medulloblastoma - EGL and neuro-epithelium (Adapted from [72]). **(F)** CECT of the spinal cord showing spinal metastases of medulloblastoma **(G)** High magnification image of invasive MB cells isolated from spinal metastases. (Adapted from [86])

1.3.2 Classification

Increasing recognition of pediatric MB as a heterogeneous disease lead to the identification of histopathological and molecular variants within MB that have distinct biological behaviors [87].

1.3.2.1 Histopathological classification

Based on the morphological and pathological characteristics of MB cells, the WHO classification (2007) of CNS tumours separated MB into five variants (Figure 4A) [88]:

- Classic medulloblastoma
- Desmoplastic / nodular (D/N) medulloblastoma
- Medulloblastoma with extensive nodularity (MBEN)
- Anaplastic medulloblastoma
- Large cell medulloblastoma

Classic medulloblastoma contain sheets of monotonous small cells with a high nuclear:cytoplasmic ratio and round nuclei. Classic MB may demonstrate moderate

cytological pleomorphism with elongated cells or oval nuclei [89]. Rosettes or palisades of cells may be present in some classic MB, the former being regarded as a sign of differentiation, though not necessarily prognostic [90].

Nodules of differentiated neurocytic cells and internodular desmoplasia are distinguishable features of D/N MB and MBEN. In MBENs, nodules often dominate the histopathology and are typically large and irregularly shaped, containing monomorphic neurocytic cells that often demonstrate linear (streaming) patterns. Nodules of uniform neurocytic cells usually round and scattered across desmoplastic regions are more evident in D/N MB [91].

As D/N MB and MBEN share fundamental histopathological features, so do anaplastic and large cell MB. Groups of uniform large cells with round nuclei and single nucleolus exemplify large cell MB. Rarely, a 'pure' large cell MB is dominated by such cells, mostly mixed populations of large cell or anaplastic cells are observed [92, 93]. Anaplasia in MB is marked by cytological pleomorphism; nuclear pleomorphism in particular. Because of very high nuclear:cytoplasmic ratio in anaplastic MB, cell molding and cell wrapping accompany nuclear pleomorphism. Both anaplastic and large cell MB shows increased mitotic activity and apoptosis [94].

For the first time, this scheme of histology based WHO classification of MB variants has clinical utility. MBENs and D/N MB in infants have a better outcome than classic tumours, while large cell and anaplastic MB behave aggressively [69, 88, 95].

1.3.2.2 Molecular subgroups of medulloblastoma

Recent multiple independent efforts at profiling the MB transcriptome have led to the discovery and description of distinct molecular subgroups of the disease [96]. Compilations of several studies around the globe have suggested the existence of multiple distinct subgroups of MB that differ in their demographics, transcriptomes, somatic genetic events and clinical outcome [97]. The four principal subgroups of MB were named as follows (Figure 4B):

- Wnt subgroup
- SHH (Sonic Hedgehog) subgroup
- Group 3
- Group 4

The Wnt and SHH were named for the signaling pathways thought to play prominent roles in the pathogenesis of that subgroup. Since less is known about the biology of the remaining two subgroups, there are collectively referred as non-Wnt / non-SHH tumours.

Wnt Subgroup: The best known of the MB subgroups is the Wnt Subgroup due to its very good long-term prognosis in comparison to other subgroups. The gender ratio for Wnt MB is about 1:1 (male:female) and can occur in all ages, but is uncommon in infants [98]. Germline mutations of the Wnt pathway inhibitor *APC* predispose to Turcot syndrome, which includes

a proclivity to medulloblastoma [99]. In addition, somatic mutations of *CTNNB1* encoding β-catenin have been found in sporadic MB [100]. These strong germline and somatic genetic data strongly support an etiological role for canonical Wnt signaling in the pathogenesis of this group of tumours. Nearly all of the Wnt medulloblastoma studied to date have a classic histology [101]. Frequent descriptions for Wnt MB include mutations in *CTNNB1* mutations, immunohistochemical nuclear staining for β-catenin and monosomy six (deletion of one copy of chromosome 6 in the tumour). There is no gold standard of these markers for the diagnosis of Wnt medulloblastoma [102]. Controversially, MB with large cell / anaplastic histology (generally represent aggressive outcome) have also been reported in the Wnt subgroup, although they appear to maintain the excellent prognosis associated with the Wnt subgroup [101].

SHH Subgroup: In this subgroup, Sonic Hedgehog Signaling pathway is thought to drive tumour initiation in many if not all cases. SHH MB has a gender ratio of approximately 1:1 (male:female) and the temporal incidence of human SHH is curiously dichotomous. It is very frequent in both infants (0-3 years) and adults (>16 years), but much less frequent in children (3-16 years) [103]. Individual with germline mutations in the SHH receptor *PTCH* have Gorlin syndrome, which includes a predisposition to SHH MB [104]. Infantile SHH MB is particularly caused in individuals with germline mutations of the SHH inhibitor *SUFU* [105-107]. Similar to germline SHH MB, sporadic SHH MB is caused due to mutations of *PTCH, SMO and SUFU* as well as amplifications of *GLI1* and *GLI2* [108]. SHH MB has largely been identified on the basis of transcriptional profiling. Other approaches to identify SHH MB include immunohistochemical staining for SFRP1 [109] or GAB1 [110] and deletion of chromosome 9q [98]. Deletion of chromosome 9q appears to be specific for SHH MB, which is appropriate as the *PTCH* gene is located at chromosome 9q22. Most, if not all D/N MB belong to SHH subgroup. However, it is not an effective marker for the subgroup as up to 50% of SHH MB are not nodular/desmoplastic [64]. The prognosis of SHH MB is similar to group 4 and appears intermediate between Wnt MB (good) and group 3 MB (poor) [97]. Nonetheless, SHH tumours with *TP53* mutations have treatment failures and a dismal prognosis [111, 112].

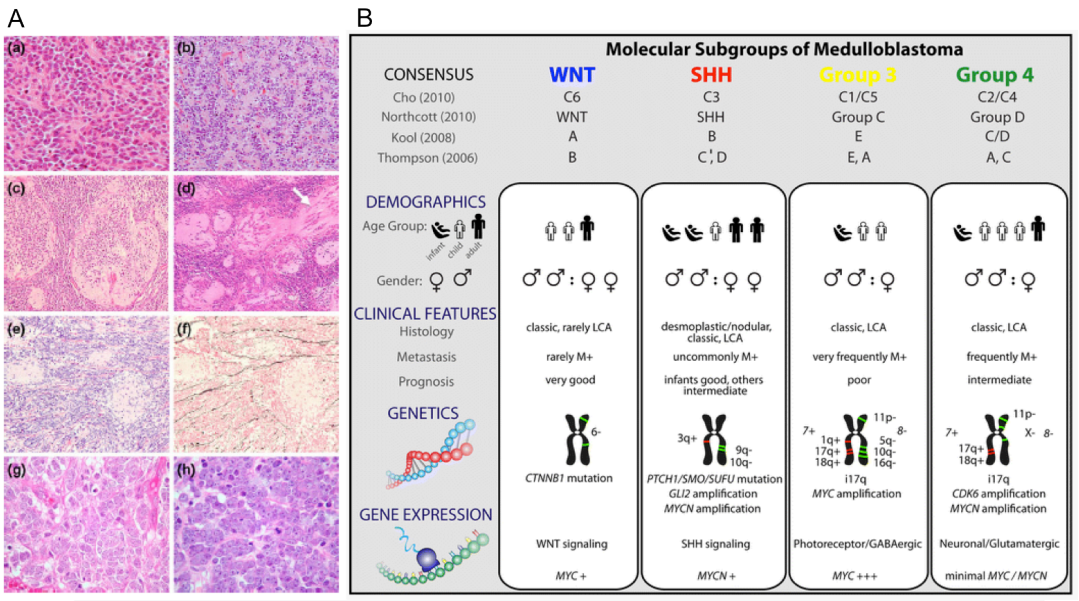
Group 3: Group 3 tumours occur more commonly in males than in females and are found in infants and children, but are almost never observed in adults [98]. SHH MB has high levels of expression of *MYCN* and Wnt subgroup and group 3 tumours have high levels of expression of *MYC,* whereas group 4 tumours have relatively low expression of both *MYC* and *MYCN* [102]. Group 3 MB can also be referred to as *MYC* group as *MYC* amplification (but not *MYCN* amplification) almost exclusively occurs in group 3 MB [98]. The current gold standard for diagnosis of a group 3 tumour is a transcriptional profile that clusters with other group 3 MB. Other approaches to identify group 3 MB include immunohistochemical positivity for NRP3 and amplification and over-expression of MB oncogene *OTX2* [113-115]. Group 3 MB are mostly 'classic' MB although they do encompass the majority of large cell anaplastic histology and are very frequently metastatic [98]. The best evidence for a clear 'subset of a subgroup' in medulloblastoma to date is found in group 3. Group 3α includes all patients with *MYC* amplifications, which assume that most of the high-risk of recurrence and death

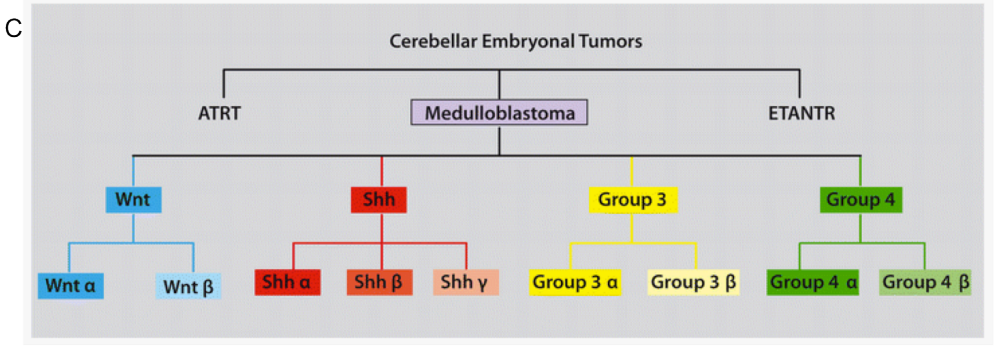
associated with group 3 diagnosis. Group 3β harbors no *MYC* amplification and had a clinical outcome similar to group 4 (Figure 4C) [102].

Group 4: Group 4 MB are prototypical MB with a classical histology that has an isochromosome 17q [98]. Although, isochromosome 17q is also seen in group 3 MB (26%), it is much more common in group 4 where it is the most usual cytogenetic change observed (66%) as reported in meta-analysis by *Kool et al.,* [116]. A peculiar and notable cytogenetic change among group 4 tumours is the loss of the X chromosome, which is seen in 80% of females with group 4 MB. The high incidence of X chromosome loss in females with group 4 MB is particularly poignant in light of the high male:female ratio (2:1) in group 4 patients [97, 98]. Currently, group 4 MB are identified via transcriptional profiling that cluster with other group 4 MB. KCNA1 has been recommended as an immunohistochemical marker for group 4 MB but it need further validation [117]. Group 4 patients have an intermediate prognosis similar to the SHH MB patients. Group 4 makes > 30% of all MB, but neither the genetic basis nor the clinical relevance is not yet apparent.

1.3.2.3 Alternative approaches for classification of Medulloblastoma

Immunohistochemistry: Despite MB being classified both at histological and molecular level, attempts are made to distinctly and easily classify MB using other methods. A study with a large cohort of MB patients (n=235) validated a novel immunohistochemical method to distinguish the molecular subgroups of MB and detailed their association with clinical, pathological and cytogenetic variables (Figure 4D) [118]. Expression of four immunohistochemical markers (chosen based on its potential role in SHH and Wnt signaling pathways and role of primary cilia in MB): GAB1, β-catenin, filamin A and YAP1 were studied. Immunoreactivity for GAB1 proved to be a characteristic feature of SHH tumours while nuclear β-catenin staining was exclusive for WNT tumours. Immunohistochemical positivity for filamin A and YAP1 was found in SHH and WNT tumours. Non-WNT / SHH tumours displayed cytoplasmic, but not nuclear immunoreactivity for β-catenin. Tumours in this category were immunonegative for GAB1, YAP1 and fliamin A. This method indicates the value of combining clinical, pathological and molecular variables in therapeutic stratification of MB.





D

Molecular group	Immunoreactivity			
	GAB1	β-catenin	Filamin A	YAP1
SHH	Cytoplasmic	Cytoplasmic	Cytoplasmic	Nuclear + cytoplasmic
WNT	Negative	Nuclear + cytoplasmic	Cytoplasmic	Nuclear + cytoplasmic
Non-SHH/WNT	Negative	Cytoplasmic	Negative	Negative

Figure 4: Classification of medulloblastoma: (A) Histological variants of MB: (a) classic MB, (b) classical MB interspersed with rosettes or palisades of cells, (c) desmoplastic / nodular MB, (d) nodular MB with large areas of nodular formation and restricted desmoplastic intermodular regions, (e) classic MB with nodules of neurocytic cells, (f) classic MB without desmoplastic regions, (g) anaplastic MB, (h) large cell MB. (Adapted from [119]). **(B)** Molecular subgroups of MB according to the current consensus. **(C)** Dendrogram depicting the subsets within the subgroups of MB. (Adapted from [97]). **(D)** Immunohistochemical classification of MB. (Adapted from [118]).

DNA methylation patterns: Although MB is distinctly classified into 4 major subgroups, constant efforts are being made to sub-classify this heterogeneous disease in order to better stratify the patients in a clinical setting. As a step towards sub-classification of MB, tumour DNA methylation profiles were assessed in 230 MB patients primarily from the SIOP-UKCCSG PNET3 clinical trials [120]. Indeed, epigenetic DNA methylation patterns across MB samples demonstrated that MB comprises four robust DNA methylation subgroups (WNT, SHH, G3 and G4), highly correlated to their transcriptomic counterparts. WNT patients displayed an expected favorable prognosis, while outcomes for SHH, G3 and G4 were equivalent as determined by the methylation patterns. In addition, MXI1 and IL8 methylation were identified as novel independent high-risk biomarkers in non-WNT patients [121]. Incorporation of MXI1 and IL8 methylation patterns into the current survival model

significantly improved the risk assessment of the disease. 46% of patients could be classified as 'favorable risk' (>90% survival) compared to 13% using current molecular sub-group models, while high-risk group was reduced from 30 to 16% [120, 122]. Thus, DNA methylation patterns could be an alternative approach for robust sub-classification of MB. Sub-group specific DNA methylation biomarkers can significantly improve current risk-stratification schemes and can be clinically implicated for risk-adapted disease management of MB.

1.3.2.5 Uses of Medulloblastoma classification system

Enormous efforts have been devoted to classify and subgroup MB. This classification and subgrouping is imperative to improve prognosis and to reduce treatment related side effects. Multiple deregulated signaling pathways and cytogenetic aberrations have been identified in MB; however, many questions about the pathogenesis of the disease remain unanswered. Hence, establishment of sub-group specific MB cell lines and mouse models will prove to be a very effective tool for answering the open questions with regard to MB pathogenesis.

Medulloblastoma cell lines: Currently, there are around 44 continuous MB cell-lines established over a period of four decades. Less than half (18/44) established MB cell-lines have been sub-grouped. The majority of the sub-grouped cell lines (11/18, the so-called MedD cell lines, e.g. D341, D425) are group 3 with *MYC* amplification. The next most common are the SHH cell-lines (4/18, e.g. DAOY, UW228), half of which exhibit TP53 mutation. Wnt and group 4 subgroups, accounting for 50% of patients, remain underrepresented with 1 and 2 cell-lines respectively [123].

Mouse models of medulloblastoma: Any basic cancer biology or translational cancer research will have genetically engineered mouse models or xenografts passaged in mouse as indispensable tools [124]. The vast majority of published mouse models of MB belong to the SHH MB. Majority of the SHH mouse models are generated by the heterozygous deletion of *PTCH* (as homozygous deletion is lethal) [125]. Mouse models with constitutively activate *SMO* [126] and TP53 mutation mouse models also represent the SHH MB [127]. Apart from the genetically engineered mouse models, non-genetically engineered mouse models involving ectopic flank and orthotopic intracranial xenografts into immune-compromised mice, such as Nu/Foxn1/Nu and NOD/SCID, have been widely used in MB research, especially for validation studies and preclinical research [128]. The non-genetically engineered MB mouse models usually represent group 3 MB that engages orthotopic injection of group 3 MB cells. Mouse models of Wnt MB suggest that Wnt MB arise from the lower rhombic lip of cerebellum [129]. No mouse models of group 4 have been reported so far.

1.3.3 Epidemiology, survival and recurrence

Epidemiology: Approximately 500 children are diagnosed with MB each year in the US accounting for 15-20% of all primary tumours of the CNS among children less than 19 years

of age. Although, MB is prevalent in children, it can also occur in patients > 21 years of age. It is estimated that MB affects 9.6 children per million and 0.54 adults per million. The peak incidence is between 5 and 9 years of age. 70% of patients are diagnosed before the age of 20 [130]. The SEER study consisting of 633 diagnosed cases of MB, demonstrated a striking 23% increase in incidence from 4 per 10^6 person-years in 1973-77 to 4.9 per 10^6 person-years in 1993-98. There is a slight increase in incidence between the ages of 20 to 24, and the disease is rare after the fourth decade, coherent with its embryonal origin. There was an overall male predominance (2:1) in MB incidence [131]. A CBTRUS study on incidence trends of MB reported no significant difference in MB incidence over the last 2 decades, but there was an increase in MB and PNET combined [132].

Survival: Recently reported 5-year overall survival rates for both children and adults are in 60^{th} and 70^{th} percentile, which is a dramatic increase from 29% 5-year overall survival reported in earlier periods [133]. Progression free survival for all patients was 68% at 3 years and 62% at 5 years [130]. The marked increase in overall survival and progression free survival suggests that salvage therapy, usually with combination chemotherapy is of benefit in this cohort of patients [134].

Recurrence: Recurrence in MB is a therapeutic challenge because it is almost always fatal. A retrospective study with 3 large non-overlapping cohorts (Cohort 1: recurrent MB at the Hospital for Sick Children, Toronto, Canada; Cohort 2: recurrent MB samples from 13 centers worldwide, obtained between 1991 and 2012; Cohort 3: recurrent MB samples obtained at NN Burdenko Neurosurgical Institute, Moscow, Russia between 1994 and 2011) using nanoString analysis identified distinct sub-group specific recurrence patterns in MB. Recurrence patterns were analyzed based on the anatomical site of recurrence (local tumour space or leptomeningeal metastasis), time to recurrence and survival after recurrence in a sub-group specific manner. Local recurrences were more frequent in SHH subgroup and metastatic recurrences were more common in group 3 and group 4 sub-groups. Survival after recurrence was significantly longer in patients with group 4 tumours than with other sub-groups [135].

1.3.4 Genomic landscape of Medulloblastoma

Consistent with the genomic landscapes of pediatric tumours, MB has one of the lowest mutation frequencies among all pediatric cancers with less than 0.1 mutation/Mb DNA (Figure 2) [27]. High-density microarrays and deep- sequencing in a set 22 MBs, found that, on average, each tumour had 11 gene alterations, fewer by a factor 5 to 10 than in adult solid tumours [136]. Naturally and predictably, most gene alterations were found in the driving pathways of MB, namely the SHH and Wnt signaling pathways (Figure 5). In the SHH pathway, *PTCH1* was mutated in 17% of the tumours and in Wnt pathway; *CTNNB1* was mutated in 13% of the tumours. Mutations in PTCH1 and CTNNB1 are predominantly germline but it could also occur sporadically [137, 138]. In addition to SHH and Wnt signaling, pathways most highly enriched for genetic alterations have not been implicated in MB. These involve genes responsible for chromatin remodeling and transcriptional regulation, the histone-lysine N-methyltransferase *MLL2*, in particular. 20% of MB tumours

harbor mutations in *MLL2* and the related gene member *MLL3*. A mix of missense, nonsense and frameshift mutations were observed in *MLL2* and *MLL3* [136]. The overall absence of other distinct mutations in MB demonstrates the key differences between the genetic landscapes of adult and childhood cancers. This also highlights deregulation of developmental pathways as an important mechanism underlying MB pathogenesis. Although, only 16% of MB patients have *MLL2* and *MLL3* mutations, these unique MB patient populations are not to be ignored [139]. The connotation of these small fractions of mutations in MB treatment and management needs to be investigated further.

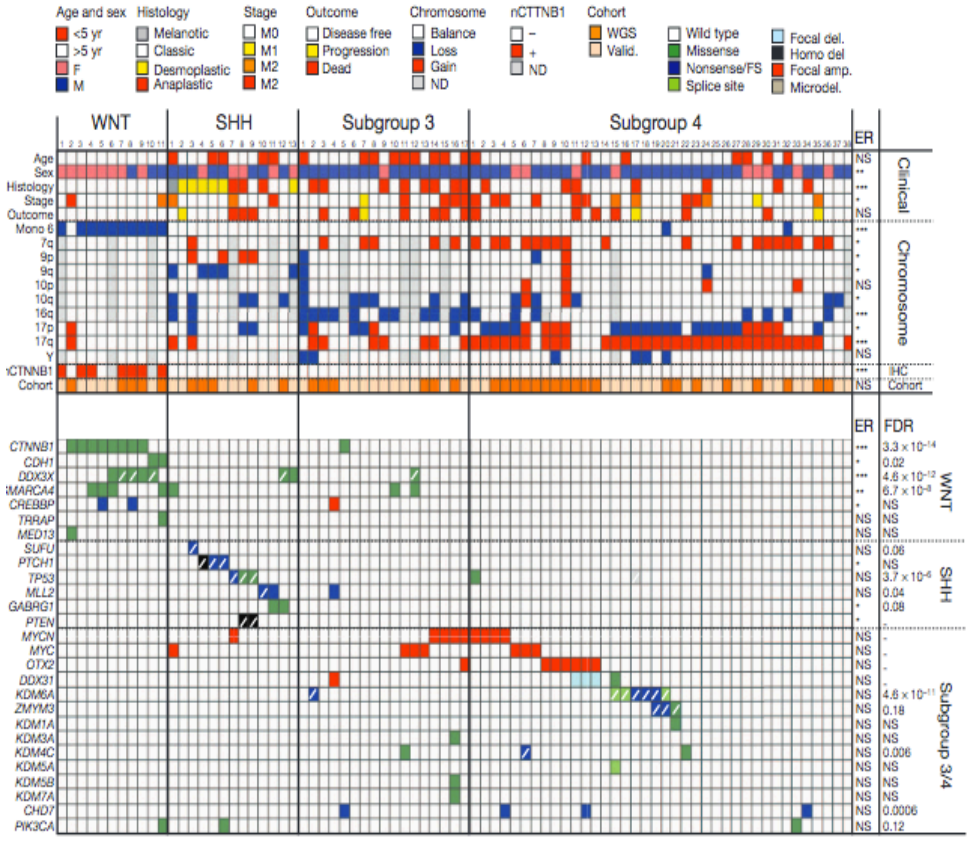


Figure 5: The genomic landscape of medulloblastoma: Clinical, histological, gross chromosomal, nuclear and cohort details of 79 MB samples by subgroup (Top). Genetic alterations detected in selective 27 genes of interest in MB (Top). (Adapted from [139])

1.3.5 Treatment modalities and late effects

Analogues to other pediatric cancers, the treatment regimens for MB also include a combination of surgery, radiation therapy and chemotherapy.

Treatment: MB constitutes 18% of the intracranial tumours and the first line therapy for MB is surgery. But due to the primary site and local infiltration, curative surgical excision is rarely possible [140]. Therefore, radiation therapy is employed as an extensive treatment modality in MB. Since this tumour disseminates malignant cells throughout the subarachnoid space via the cerebrospinal fluid, radiation therapy is administered to the entire CNS [141]. With the combination of surgical resection and maximally tolerated CNS axis radiation therapy (A dosage of 27 to 30Gy to the cranium and cervical cord administered in fractions over 4

weeks and a 2 week boost of 15 to 20Gy to the posterior fossa), there is a dramatic increase in the overall survival rates. Post-operative craniospinal radiotherapy results in a 5-year survival between 55 and 70% of all stage groups [142].

Accompanying the improvement in long-term survival, there is an increasing concern about the late effects of late-effects associated with radiotherapy such as hormonal deficits, decreased bone growth, hearing loss, neurocognitive defects, cardiac dysfunction, pneumonitis, stenosis of esophagus and secondary cancers [142]. Radiotherapy related late-effects are reduced by the following strategies in the clinics:

- **Intensity / energy modulated proton therapy:** Proton therapy uses a beam of protons rather than x-rays to irradiate the malignant cells and cancerous lesions. Proton therapy allows the delivery of a high dose, highly focused radiation beam precisely to the tumours site [143]. Up to 60% less radiation can generally be delivered to the normal tissue around the tumour, thus reducing the late-effects [144]. A recent comparative treatment planning study reported that proton therapy in MB reduces the risk of development of secondary cancers by 4% [145].
- **Adjuvant chemotherapy:** MB is a classic example where chemotherapy is used as an adjuvant therapy in an attempt to delay or avoid radiotherapy in infants (0-3 years) and to reduce late-effects in children (3 to 19 years) [48, 146]. Chemotherapy cannot be used as a monotherapy in MB as it is toxic and less effective [147]. Children with standard risk are treated with radiotherapy alone. High-risk patients (high inclination for disease relapse) are treated with standard craniospinal radiotherapy with adjuvant chemotherapy consisting of vincristine during radiotherapy followed by 6-week cycles of 1-(2-chloro-ethyl)-3-cyclohexyl-1-nitrosourea (CCNU), vincristine and cisplatin [148] [149]. Recently methotrexate [150] and temozolomide [151] are also used in MB adjuvant chemotherapy, as they are less toxic and well tolerated. Pre-irradiation chemotherapy (neo-adjuvant chemotherapy) causes poorer survival of MB patients, due to the increased myelotoxicity as compared to post-irradiation chemotherapy [152].

Taken together, treatment protocols for MB essentially depend on risk-stratification of patients and molecular sub-group of MB [149]. As most patients with Wnt MB survive, there is a possibility that they are being over treated with current therapies, which causes high morbidity and there is an active discussion of a clinical trial of therapy de-escalation in this patients [97].

Late effects: Posterior fossa syndrome (PFS) is one of the most common late effects of any intracerebellar tumours, MB in particular. Complete surgical excision of tumour is the standard of care in the treatment of non-disseminated MB, which advocates for aggressive surgical resection. PFS is a debilitating consequence of posterior fossa surgery in children with MB. PFS manifests initially with mutism that occurs after a latent interval of 1 to 2 days following surgery. Most children experience a variable period of subsequent dysarthria prior to normal speech recovery. Ataxia and hypotonia are common features that coincide with the development of speech deficits [153].

Neurocognitive late effects in MB survivors are relatively more common and can be highly incapacitating [154]. The sequelae of radiation therapy impact the neuropsychological functioning of the brain in MB patients [155]. Higher doses of radiation positively correlate with lower IQ in MB survivors [156]. Survivors of MB may suffer from neurological impairment with pain, seizures and sensory loss [157]. With regard to the neuroendocrine late effects, growth hormone deficiency is the most frequently noted endocrinopathy in MB [158]. Most survivors develop growth hormone deficiency relatively early after treatment [159]. Other endocrinopathies include hypothyroidism [160] and sex hormone dysfunction [160]. Intensive surgical resection and radiotherapy leads to the formation of secondary malignancies in MB [161].

1.3.6 Targeted therapies in medulloblastoma

Targeted therapies pertinent to medulloblastoma are still in its pre-infancy stages. Till date, there are no FDA approved targeted therapies for medulloblastoma. This, in part can be attributed to the advances in the current treatment regimens, which has dramatically improved the 5-year event free survival. According to the most recent statistics, the five year event free survival rates for MB patients with high-risk MB are > 60% and for those with standard-risk are >80% [130]. Despite this high survival rates, patients with high risk of recurrence (aged <3 years, with significant residual disease) have poor survival rates and long-term control in these patients are difficult to achieve. In addition, patients with high-risk and recurrent tumours have to face a paucity of stringent treatment regimens, which are most often associated with long-term toxicities [162]. Considering the lack of a salvage therapy that is clearly effective and durable for recurrent MB, it is evident that novel targeted therapies are needed for patients with MB.

The necessity to develop novel targeted therapies coupled with the molecular subgroups of MB has sparked numerous pre-clinical and clinical studies of molecularly targeted therapies in MB models. Since MB is subdivided into four subgroups based on the transcriptional profiling and aberrant signaling pathways activated, these clinical studies could be classified as therapies targeting signaling pathways directly linked to MB and therapies targeting additional signaling pathways and cellular processes indirectly associated with MB.

1.3.6.1 Clinical investigations of targeted therapies directly linked to MB

Hedgehog inhibitors: Constitutive activation of hedgehog signaling – often due to inactivating mutations of *PTCH1* has been shown in approximately 30% of MB in human [97]. Numerous pre-clinical and clinical observations have shown that small molecules targeting smoothened (SMO) are highly effective, albeit temporarily, against SHH MB [163]. GDC-0449 (Vismodegib) is an orally bioavailable selective inhibitor of SMO (FDA approved in 2012 for treatment of basal cell carcinoma) that induces rapid regression of the tumour and suppression of the hedgehog pathway. A 26-year MB patient enrolled in the phase 1 trial with GDC-0449 showed complete remission at the beginning of the therapy but developed resistance after 2 months of treatment with GDC-0449 [164]. Identifying the mechanism of

acquired resistance to selective hedgehog pathway inhibitors in patients with MB will be of particular interest in future studies.

In contrast, LDE225, a potent second-generation SMO inhibitor is currently being evaluated as a monotherapy in pediatric and adult patients with recurrent or refractory MB. LDE225 is well tolerated and showed anti-tumour responses in phase 1 study. All the patients who responded to LDE225 in the study cohort had a signature pattern of SHH activated tumours [165]. Therefore, several additional trials are currently ongoing, including a phase 3 trials in relapsed MB with activated SHH pathway. Although, these clinical results seems promising, MB can develop resistance against SMO inhibitors as SMO and GLI are frequently mutated in MB. Several preclinical studies have been designed to circumvent the resistance such as combination of LDE225 with other pathway inhibitors [166].

WNT pathway inhibitors: WNT subgroup of MB has one of the best prognoses and the molecular mechanisms driving this subtype of MB is well characterized. This led to the identification of various agents targeting the WNT pathway, such as PARP5/tankyrase inhibitors olaparib and veliparib (ABT-888). Currently there are no clinical trials with WNT pathway inhibitors for MB. However, these inhibitors are being evaluated in pediatric patients with CNS tumours [162].

1.3.6.2 Clinical investigations of targeted therapies indirectly linked to MB

Notch inhibitors: MK-0752, an inhibitor of γ-secretase showed promising results in pre-clinical mouse models but did not fair well in the clinical trials with a wide variety of nervous system tumours including MB [167].

PI3K/AKT/mTOR and RAS/MEK/ERK inhibitors: Currently, no clinical trials are evaluating PI3K and MEK inhibitors in MB. However, these inhibitors have been tested in patients with CNS tumours. Sirolimus, an mTOR inhibitor is now in phase I trials in combination with celecoxib (COX-2 inhibitor) and etoposide, alternating with cyclophosphamide for relapsed or refractory solid tumours including MB [162].

EGFR inhibitors: Ongoing clinical trials are testing erlotinib in combination with chemotherapy and radiotherapy in children with CNS tumours. Data from these trials show that erlotinib combined with temozolomide is well tolerated in pediatric patients. Although no objective response was observed, the disease remained stable in MB patients with this treatment regimen [168].

1.3.6.3 Clinical investigations of additional cellular processes in MB

Anti-angiogenic approaches: Like EGFR inhibitors, inhibitors against VEGF and PDGF are also being evaluated in combination with standard chemotherapy or other targeted agents in MB patients. MB patients treated with a combination of bevacizumab, thalidomide, celecoxib, fenofibrate, etoposide and cyclophosphamide exhibited favorable rates of event-free survival [169, 170].

COX-2 inhibitors: Celecoxib when combined with temozolomide increased the clinical stability of disease and achieved an objective response in pediatric patients with replapsed MB. Following these promising results, celecoxib is now in clinical trials in combination with anti-angiogenic agents for patients with recurrent or progressive MB [171].

Histone deacetylase (HDAC) inhibitors: Clinical trials with the HDAC inhibitors, demonstrated that vorinostat (HDAC inhibitor) is well tolerated both a monotherapy and in combination with isotretinoin in pediatric patients with recurrent / refractory solid tumours including MB [172].

Alkylating agents: Temozolomide is a recent addition to the standard treatment regimens for MB patients. Several clinical trials have tested the efficacy and safety of temozolomide as a single agent and in combination with other chemotherapeutic agents and radiation therapy [173]. Temozolomide has a synergistic effect when with etoposide and this combination exhibited favorable outcome in MB patients in the clinical trials [174]. Similar finding were documented with the use of the platinum-based alkylating agent, namely carboplatin, a well know potent anti-cancer agent. Initial results from the trials with its synergistic combination with temozolomide are very promising with evidence of 5-year progression free survival of 53% in MB patients. In addition, carboplatin also synergizes with etoposide and methotrexate. MB patients treated with carboplatin and etoposide had a response rate of 72% while the patients treated with a triple combination (carboplatin, etoposide and high dose methotrexate) had a response rate of 74% [175, 176].

Topoisomerase inhibitors: Etoposide is not effective as a monotherapy for MB patients as is failed to reverse stable disease progression. However, as described earlier, it can synergize with carboplatin and in combination with craniospinal radiation, it can achieve a 5 year overall progression free and overall survival rate of 57.6% and 80% respectively [177].

Microtubule inhibitors: With an assortment of microtubule inhibitors (paclitaxel, docetaxel, vinorelbine) approved by FDA, an overwhelming number of clinical trials have evaluated these compounds for the treatment of MB. Intravenous daily administration of paclitaxel showed a complete response in one MB patient and stabilized the disease in other MB patients included in the trial [178]. Unlike paclitaxel, docetaxel and vinorelbine demonstrated only a modest success in clinical trails for the treatment of MB patients. Moreover, microtubule inhibitors often cause severe adverse side effects, which hampers its effective translation into the clinics [179].

1.4 Tumour microenvironment

Cancers develop in complex tissue environments, which they depend on for sustained growth, invasion and metastasis.

1.4.1 Role of the tumour microenvironment in cancer invasion and progression

Genetically abnormal cells define the tumour compartment itself and the epithelial parenchyma of carcinomas, the surrounding and interwoven stroma, provides the connective tissue framework of the tumour tissue [180]. This framework includes a specific type of extracellular matrix (ECM), the tumour matrix, as well as components such as fibroblasts, immune and inflammatory cells and blood-vessel cells [181]. Sandwiched between the tumour epithelia and tissue framework is the natural barrier basement membrane (BM) [182]. Cancer development and progression is a product of an evolving crosstalk between different cell types within the tumour and its surrounding tissue microenvironment (also called tumour stroma) [183, 184].

Cancer cells themselves can alter their adjacent stroma to form a permissive and supportive environment for tumour progression [185]. Most of the cancer cells prototypically generate a supportive microenvironment by producing stroma-modulating growth factors and cytokines. These factors disrupt normal tissue homeostasis and act in a paracrine manner causing the activation of stromal cells such as fibroblasts, smooth-muscle cells and adipocytes (so-called 'reactive' or 'activated' stroma) (Figure 6). The activated stromal cells secrets additional growth factors and proteases eventually promoting stromal reactions such as angiogenesis and the inflammatory responses [186]. Concomitant with the 'activated' stroma, the cancer cells produce proteolytic enzymes. These proteolytic enzymes, commonly grouped under the family of proteases called 'Matrix metalloproteinases (MMPs), degrade and remodel the ECM and basement membrane to allow for a pro-migratory and pro-invasive environment [187]. The MMPs are a family of zinc-containing endopeptidases, which collectively cleave most (if not all) of the ECM proteins. Proteolysis of the ECM is the crucial first step for tumour cell invasion [188]. Following this, the invaded cells spread and penetrate connective tissue barriers, basement membrane, provision matrix or interstitial stroma and ultimately extravasate, metastasize and form secondary tumours [184].

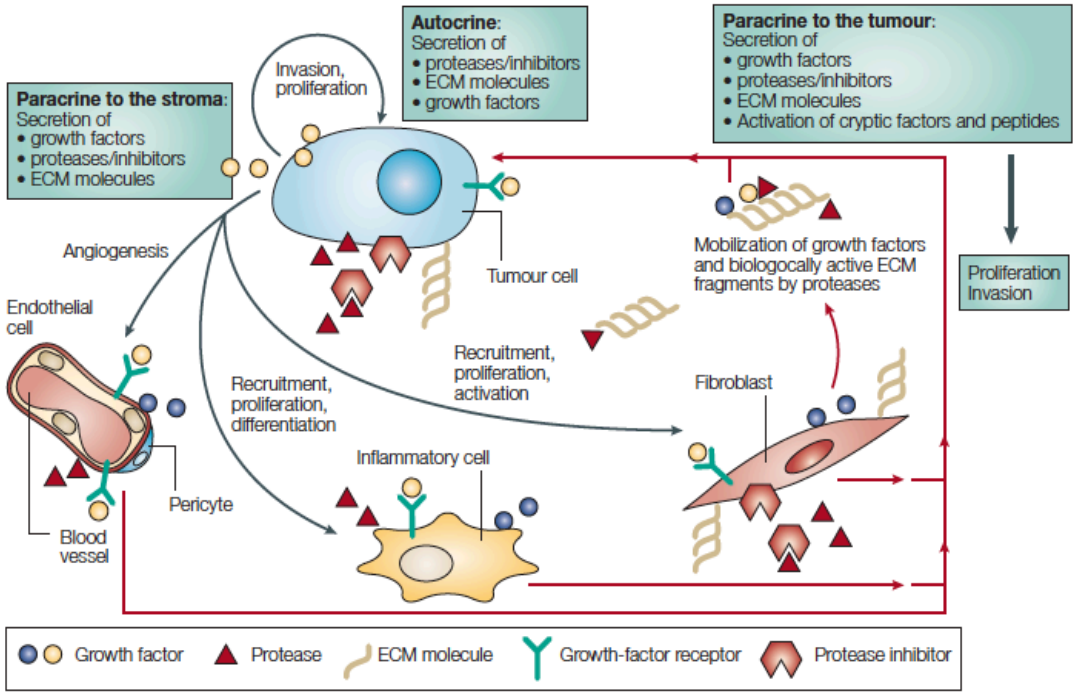


Figure 6: Schematic representation of crosstalk between tumour cells and their activated stromal surroundings. (Adapted from [186]).

1.4.2 Extracellular matrix

ECM is the non-cellular component of the tumour microenvironment, constituting approximately 60% of the total tumour-stroma niche.

1.4.2.1 Extracellular matrix components

The ECM is composed of a large collection of biochemically distinct components including proteins, glycoproteins, proteoglycans and polysaccharides with distinguishable physical and chemical properties [189]. Structurally, these components compose both the basement membrane and the interstitial matrix of the stromal cells. Basement membrane is a specialized ECM, which is more compact and less porous than interstitial matrix. It has a distinctive composition of laminins, type IV collagen, fibronectin and linker proteins such as nidogen and entactin (protein which connect collagens with other protein components) [190]. The highly charged and hydrated interstitial matrix is rich in fibrillar collagens, proteoglycans and various glycoproteins such as tenascin C and fibronectin, and greatly contributes to the tensile strength of tissues [190-192].

The aforementioned constituents of basement membrane and interstitial matrix can be groups into 2 main classes of macromolecules: proteoglycans and fibrous proteins. Glycosaminoglycan chains covalently linked to a specific protein core (except hyaluronic acid) forms proteoglycans, which fills the majority of the extracellular interstitial space within the tissue. The main fibrous ECM proteins are collagens, elastins, fibronectins and laminins. Collagens are major constituents of both basement membrane (type IV collagen) and interstitial matrix ECM (type I collagen), representing as much as 30% of total

mammalian protein mass [193]. Collagens in ECM provide tensile strength, regulate cell adhesion, support chemotaxis and migration and direct tissue development. Fibroblasts that reside in the stroma predominantly transcribe and secrete collagen and align collagen fibers into sheets and cables.

Collagen associates with elastin; another major ECM fiber protein, which is responsible for providing recoil to tissues that undergo, repeated stretch. Its tight association with collagen fibers crucially limits elastin stretch [194].

Fibronectin is intimately involved in directing the organization of the interstitial matrix and has a vital role in mediating cell attachment and function [195]. Fibronectin is important for development-associated cell migration [196]. Like fibronectin, other ECM fiber proteins like tenascin exert pleiotrophic effects on cellular behavior, including the promotion of fibroblast migration [197]. Indeed, levels of tenascins C and W are elevated in the stroma of some of the transformed tissue where they promote tumour growth and metastasis [198].

1.4.2.2 Extracellular matrix receptors

Adhesive interactions between tumour cells and the insoluble meshwork of ECM happen through the ECM receptors. The ECM receptors act as a liaison between the tumour cells and ECM, whereby the signals from the ECM are transduced into the intracellular machinery that controls cell growth, migration and differentiation [199].

Integrins, a major family of ECM receptors, are heterodimeric surface receptors composed of α and β chains [200]. Together these chains mediate adhesion and mechanotransduction to extracellular ligands, including α2β1 integrin predominantly binding to fibrillar collagen; αVβ3, αVβ1 and α5β1 interacting with fibronectin; and α3β1 and α6β1 engaging with laminin [200]. After binding with ligands, the cytoplasmic tails of the integrins connect to the cytoskeletal adaptor proteins such as talin, paxillin and kindling and the mechanosensing modulators like vinculin and p130Cas [201, 202]. Adaptor proteins and mechanosensing modulators alter the actin cytoskeleton and trigger signaling to protein kinases including focal adhesion kinases (FAK) and Src kinase [200, 203]. Integrins are also shown to signal through small GTPases Rac and Rho [203].

CD44 and its alternatively spliced variants bind to hyaluronic acid (a high-molecular weight glycosaminoglycan abundantly present in all connective tissue) and with low affinity to heparin sulphate, collagen and fibronectin [204]. CD44 links itself to the actin cytoskeleton via the adaptor proteins ezrin, radixin and moesin (ERM) and ankyrin and mediates intracellular signaling through Src kinase and RhoA (small Rho GTPases)[203, 204]. CD44 also has the ability to bind to various growth factors and enhance their signaling through *cis* interactions with the respective growth factor receptors [205]. Thus, CD44 delivers joint ECM and growth factor signaling to tumour cells. CD44 serve as a co-receptor for integrins and podoplanin (a cell surface mucin that alters actin cytoskeleton via ezrin) [206].

Similar to CD44, membrane-bound proteoglycans such as syndecans, glypicans and neuropillin, interact through their sugar moieties weekly with ECM components, including hyaluronic acid, fibronectin, collagen or elastin [205]. These connections enhance the interactions between integrins, integrins and growth factors and via proteoglycans deliver signals via protein kinase C (PKC) and Src kinase [203].

The disoidin domain receptors DDR1 and DDR2 interact selectively with fibrillar collagen and signal through STAT5, NFκB and Mitogen Activated Protein Kinase (MAPK) / ERK or the Src-related kinases respectively [207]. DDRs support E and N cadherin mediated cell-cell adhesion and enhance proteolytic functions of MMPs in the tumour cells [208-210].

Integrins, CD44 and DDRs are responsible for cell adhesion and migration of tumour cells invading singularly. E and N cadherins transmit cell-cell adhesion forces to the actin cytoskeleton and provide cooperation between tumour cells during collective invasion [211]. Cadherins form stable cell-cell adhesions through adherent junctions via the transient co-engagement with small GTPases Rac1 and RhoA [212]. Depending on the type of tumour, different sets of cadherins are expressed such as E-, N-, P- cadherins, cadherin-11 and cadherin 13 that promote tumour cell invasion [213, 214].

1.4.3 Growth factor and cytokines in the tumour microenvironment

The tumour microenvironment is rich in polypeptide growth factors (PGF) and cytokines, a second major component of the 'active' stroma [215]. Extracellular signals exerted by PGFs plays a critical role in tightly regulating the growth and differentiation programs of various cell type, including epithelial cells in particular. Defects in such signaling cascades may circumvent the normal pathways of epithelial growth and differentiation and propels the cells towards a malignant transformation [216]. The spotlight is on the epithelial cells because of the overwhelming number of cancers with epithelial origin.

A major venue of communication between tumour cells and their microenvironment is through PGFs and receptors for these growth factors. A PGF binds to its corresponding cell-surface receptors and initiates intracellular signal cascade that leads to the modulation of gene expression [217]. The end result of PGF / cytokine action is to exert growth, survival, differentiation or migration or combinations of any of these. Different PGFs bind to different cell types in the ECM or PGF can also bind and activate signaling cascades in tumour cells. Both stromal cells and tumour cells contribute to the production of PGFs into the microenvironment. Therefore, abnormal production or abnormal cellular responses to PGFs underlie malignant transformation [218]. The following PGFs / Cytokines are unequivocally present in the tumour microenvironment of most solid tumours and are implicated in cancer invasion: Epidermal Growth Factor (EGF), Hepatocyte Growth Factor (HGF), Insulin-like Growth Factor-1 (IGF-1), Transforming Growth Factor-β (TGF-β) and cytokines include Tumour necrosis factor-alpha (TNF-α) and Stromal-cell derived factor (SDF-1) / CXCL12. Different PGFs / cytokines manipulate different signaling pathways and utilize several mechanisms to promote malignant transformation and progression [219].

EGF: The most extensively studied growth factor system for induced cell motility is the EGF and its receptor system [220]. Epidermal Growth Factor Receptor (EGFR) activating autocrine loops are present in most of the carcinomas [221]. PLC-γ is the classical immediate downstream effector of EGFR cascade that promotes cell migration [222]. Cell migration induced via EGF is dependent on PI3K and AKT activity [223, 224]. In addition, EGF can also promote cancer cell invasion by phosphorylating and inactivating integrin based cell contacts with ECM and by increasing the expression of MMPs [225].

HGF: HGF and its receptor c-Met are overexpressed or amplified in many types of cancer [226]. HGF also aptly called as 'scatter factor' actuates motility via the activation of c-Src, PI3K and PKC and by increasing the MMPs expression in the ECM [227]. In certain cancers like breast cancers and prostate carcinoma, there is a cross talk between HGF and EGF signaling cascades. Activated c-Met can activate EGFR through c-Src activation. HGF is a pro-growth factor, which requires cleavage in the extracellular milieu for its activation. The urokinase plasminogen activator (uPA) system that activates HGF is upregulated by EGFR signaling [228, 229]. Presence of somatic c-Met mutations in metastatic carcinomas further underscores the role of HGF in promoting motile-invasive phenotype of cancer cells [230].

IGF-1: IGF-1 and its receptor system promote cell motility primarily by activating AKT and MAPK pathways [231]. In addition to the above pathways, IGF-1 also subsidizes cell migration by coordinately stimulating integrins. IGF-1 binds to vitronectin (ECM protein) and this complex promotes cell migration by sustained activation of PI3K / AKT pathway [232]. Activated IGF-1 pathways leads to an increased expression of MMP-9 in the ECM, which in turn leads to enhanced cancer cell invasion [233].

TGF-β: TGF-β, a signaling system distinct from the classical growth factor receptors mentioned above, signals via serine/threonine kinases and SMAD effectors and has a pivotal role in the initial dissemination process [234, 235]. High levels of TGF-β signaling are appreciated in fast moving and intravasating cells. Transient high TGF-β activity in the primary tumour enables high metastatic efficacy and decreased TGF-β activity in the secondary site allows the resumption of cell proliferation [236]. Furthermore, TGF-β1 signaling induces ECM deposition (collagens, fibronectin, tenascin, elastin), thus creating a strong pro-migratory niche [237, 238].

Similar to PGFs, cytokines / chemokines also predominate the tumour microenvironment and are generally secreted by specific immune cells in the microenvironment. Cytokines and chemokines are responsible for inflammatory responses and they also promote cancer cell invasion [239-242].

TNF-α: Despite being identified as an anti-tumour cytokine by inducing immune-mediated necrosis, perceptible evidences indicate that TNF-α promotes cancer cell migration and invasion [243]. TNF-α signals through NF-κB and JNK signaling pathways, followed by elevation of MMP production in cancer cells [244, 245]. TNF-α is overexpressed in an array of cancers including lymphoma, breast [246], ovarian [247], pancreatic [248], renal [249], colon [250, 251] and prostrate cancers [252]. TNF-α also enhances cell migration and

metastasis through induction of chemokine receptor CXCR4 in a NF-κB dependent manner and by up-regulating endothelial lectin-like oxidized-low-density lipoprotein receptor-1 (LOX-1) in ovarian cancer and breast cancer respectively [246, 247].

SDF-1/CXCL12: CXCL12 binds to its widely expressed cell surface receptor CXCR4 [253, 254]. CXCL12 when bound to CXCR4 activates PLC and PI3K signaling cascades and inhibits adenylyl cyclase by different G protein subunits [255]. Signaling from PI3k actuates PAK, AKT and RhoGTPases, and that from PLC activates PKC and ERK, which play important roles in cell polarization and actin polymerization processes which are involved in cell migration [256, 257]. The expression of CXCR4 is subjective and is regulated by VEGF and TNF-α [247, 258]. CXCL12-CXCR4 signaling is marked by the up-regulation of MMP-13 and MMP-9 that regulate the axis of migration of cancer cells to metastatic sites [259].

As major components of the tumour microenvironments, ECM and growth factors interact with each other thus influencing the overall outcome of cancer invasion and progression. Fibronectin binding domains are prevalent in many ECM proteins and membrane receptors. Fibronectin and vitronectin bind to HGF and form complexes with c-Met or integrins, leading to an increase in cell migration [260]. Similarly, VEGF bind to a specific type of fibronectin (FN3) and tenascin-C, which promote cell proliferation [261].

TGF-β and its association with ECM proteins is one of the best-studied ECM-growth factor interactions. Precursors of TGF-β are cleaved to a mature TGF-β form, which is bound to latency-associated proteins (LAP). The LAPs are then bound to one of latent TGF-β binding proteins (LTBPs) to form large latent complexes (LLCs) and many cells secrete TGF-β already organized into such complexes [262]. Subsequently, LLCs associate with ECM proteins like fibrillins and fibronectins leading to effective activation of TGF-β [263]. The differential regulation of growth factors by various ECM proteins highlights the possibilities of differential outcome when growth factors are present in association with different matrix proteins.

1.4.4 Tumour microenvironment of brain tumours

Tumour microenvironment provides critical support for tumour growth and progression. The composition of tumour microenvironment depends on the tumour site. The tumour microenvironment of brain tumours is unusual in this aspect because of their composition with specialized cell types such as microglia, astrocytes, pericytes and brain endothelial cells. In addition to these brain-resident cells, populations of the bone marrow-derived cells also infiltrate the primary and metastatic brain tumours [264]. The role of different cell types in the tumour microenvironment of brain tumour and their contribution to tumour progression and invasion is not fully understood. However, some of the known interactions between the brain tumour cells and its microenvironment are listed below:

Blood vessels and cancer cells in the brain: Human brain being the most densely vascularized organs; brain tumours are among the best-vascularized tumours. Blood vessels in the brain significantly differ from the blood vessels in other organs with respect to their

tightness and structure. There is a significant increase in the blood vessel density of the cancer cell line growing in the brain as compared to their subcutaneous counterparts [265, 266]. In addition, there is a marked difference between endothelial cells in subcutaneous tissue and brain endothelial cells in their response to VEGF (one of the main inducers of angiogenesis). Over-expression of VEGF in cancer cells has been shown to increase angiogenesis in brain tumours but not in tumours of other origin [267]. Metastatic cancer cells, which extravasate from the blood stream and escape into the brain parenchyma, stay in a close proximity to blood vessels. The tight association of extravasated cancer cells with the blood vessels is critical for tumour growth as non-vessel associated cells have shown regress [268].

Hyperdilated and actively proliferating blood vessels are hallmark features of high-grade brain tumours and brain metastasis [269, 270]. The different mechanism of neovascularization in brain tumours include growth of cancer cells around pre-existing blood vessels (vessel cooption), sprouting of vessels, vasculogenesis (recruitment of endothelial progenitor cells from different sources such as bone marrow, existing vasculature or adipose tissue to aid in the formation of blood vessels) and differentiation of stem cells to endothelial cells which then contribute to tumour vasculature [271, 272]. Angiogenesis is principally regulated by proteases that digest the basement membrane, angiogenic factors like VEGF, bFGF, angiogenic inhibitors like trombospondin-1 and angiostain, and factors that stabilize interactions between endothelial cells and supporting pericytes like angiopoietin 1 and 2 [273] [274, 275]. Moreover, during vasculogenesis the concentrations of chemoiknes like stromal cell-derived factor -1 (SDF-a) and granulocyte-monocyte colony stimulating factor (GM-CSF) are increased thus creating an overall tumour-promoting environment in the brain [276, 277].

Pericytes: Perivascular cells responsible for supporting blood vessel growth and vascular maturation are called as pericytes [278]. Pericyte progenitor cells (PPCs) express Platelet-derived growth factor receptor B (PDGF-B) and are recruited to the brain blood vessels by endothelial cells by the secretion of PDGF-B. Pericytes recruited to the blood vessels promote growth and maturation of the brain tumour vasculature [279]. Indeed, depletion of pericytes by anti-PDGF-B antibody leads to enlargement and hyperdilation of tumour vasculature and increased apoptosis of endothelial cells [280].

Pericyte-endothelial cell interactions are maintained and controlled by the TGF-β signaling, which plays an important role during vascular development [281]. Endothelial cells secrete TGF-β, which promotes the differentiation of PPCs [282]. Reciprocal communication between endothelial cells and pericytes also involve angiopoietin-Tie2 (Key signaling involved in vessel stabilization). Endothelial cells express Tie2 receptor, whilst its ligand Ang1 is expressed on juxtaposed perivascular pericytes. Paracrine Tie2-Ang1 signaling enhances stability and maturation of blood vessels [283]. Immature NG2-expressing pericytes may also contribute to brain angiogenesis by sequestration of angiotensis, which is a known negative regulator of endothelial cell proliferation and migration [284].

Macrophages and microgila: The CNS contains different subsets of macrophages, most prominent including the parenchyma microglia and the perivascular macrophages [285, 286]. Activated / reactive microglia and macrophages are identified by increased expression of CD68 and are frequently found to infiltrate primary and metastatic brain tumours [287, 288]. The percentage of microglia / macrophages in the brain tumour microenvironment varies from 8-78% of all cells in gliomas and 4-70% of cells in brain metastases [289, 290]. Brain tumour cells secrete many factors such as macrophage chemoattractant protein 1 and 3 (MCP-1, MCP-3), G-CSF and HGF, which attracts the microglia / macrophages to the tumour site [291-294]. In turn, the microglia / macrophages secrete multiple cytokines, growth factors, enzymes and reactive oxygen species that can directly or indirectly result in angiogenesis (e.g. VEGF), tumour proliferation (e.g. EGF) and invasion (e.g. metalloproteases) [295-297].

Different myeloid lineages produce distinct factors that contribute to brain tumour invasion and progression. The so-called vascular modulatory cells of the myeloid lineage (CD11b+ CD45+) are recruited to the tumour site via SDF-1/CXCR4 axes upon the hypoxic up-regulation of SDF-1 in tumours. These cells release matrix-bound VEGF by expressing MMP-9 thereby increasing tumour angiogenesis [298]. Similarly, a subset of circulation and tumour-infiltrating Tie-2 expressing monocytes (TEM) express bFGF which accounts for the majority of proangiogenic activity of the tumour infiltrating myeloid cells in glioma [299].

In contrast to the above tumour promoting activities of microglia / macrophages, some studies have demonstrated tumour suppressive properties of microglia. Macrophages when depleted through macrophage-specific antibodies resulted in a significant increase in glioma growth, suggesting that macrophages suppress tumour growth in the brain [296, 300].

Microglia / macrophages also contribute to the immunosuppressed environment in brain tumours by secreting immunosuppressive factors like TGF-β, IL-10 and prostaglandin E2 and by down-regulating the expression of pro-inflammatory cytokines TNF-α and MHCII. This renders these cells unable to present antigens to T cells [301-304]. Immune suppression in the CNS is also maintained through the interaction between Fas and FAS ligand. Macrophages have a high expression of FAS-L, which reduces lymphocyte infiltration into the tumour site, likely due to the FAS-L induced apoptosis of Fas-expressing T-cells [305].

Astrocytes: Astrocytes are glial cells that become active upon different CNS injuries. The phenomenon of activation of astrocytes is known as 'reactive gliosis' and is characterized by cellular hypertrophy and changes in astrocyte gene expression patterns such as up-regulation of Glial Fibrillar Acidic Protein (GFAP) [306, 307].

Reactive astrocytes with its idiosyncratic increased expression of GFAP are present in the vicinity of primary and metastatic brain tumours [308]. Wide ranges of neurotropic factors and growth factors are secreted by astrocytes to promote the proliferation and survival of brain tumour cells. These include TGF-α, CXCL12, S1P and GDNF, IL-6, TGF-β and IGF-1 [297]. In addition, heparanase, an enzyme that degrades heparin sulfate proteoglycans that is produced by astrocytes contribute to the invasiveness of cancer cells in the brain [309].

Production of pro-MMP2 and uPA by astrocytes also promotes invasiveness of brain cancer cells. In this context, brain tumour cells produce plasminogen, which is processed to plasmin by uPA. Finally, plasmin converts pro-MMP2 to MMP2. Astrocytes are capable of sequestering intracellular calcium, which is implicated in apoptosis. This sequestration of calcium protects the cancer cells in the brain from chemotherapy-induced apoptosis [310].

Like microglia / macrophages, astrocytes are proficient immunosuppressors in the CNS. Astrocytes down-regulate the production of pro-inflammatory cytokine TNF-α by monocytes and microglia, and suppress the up-regulation of MHCII and CD80 in these cells. This impairs the ability of monocytes / microglia to present antigens to T cells and to promote T-cell activation [311].

Fibroblasts: Stromal fibroblasts are ascribed to play a key role in the induction of angiogenesis and metastasis when associated with brain tumours cells. Brain tumour cell related fibroblasts promote the production of MMP2 and its activators MT1-MMp and MT2-MMP, whose activation ascertains brain tumour invasion and progression [312].

Endothelial cells: Endothelial cells are the source of oxygen and nutrients for tumours cells. They are critical partners participants in the progression of brain tumours, which may be attributed to their multivariate contributions like secretion of factors that help maintain stem-like features of brain tumour cells and mediating tumour angiogenesis [264, 313].

Brian tumour stem-like cells: in contrast to the aforesaid perivascular niche cells that are not neoplastic by themselves, there are Brain tumour stem cells (BTSCs), that live in the brain tumour niche. These populations of tumour cells are resistant to therapy giving rise to recurrence and express many stem cell markers [264, 313]. The self-renewal properties of BTSCs are enhanced via niche-derived factors like nitric oxide (NO). NO activates notch signaling through which BTSCs self-renews and promotes tumorigenesis of gilomas. Further, eNOS, an enzyme that synthesizes NO from the vascular endothelial cells, is elevated in the PDGF-subset of gliomas. Subsequently, suppression of eNOS, hampered NOTCH signaling thus hindering the maintenance of BTSCs in the niche. However, it remains to be determined if NO signaling and its contribution towards the maintenance of BTSCs is conserved across other brain tumour sub-types [314].

1.4.5 Clinical implications and targeting tumour microenvironment for cancer therapy

Abundant evidences show that 'active / reactive' tumour stroma is required for and contributes to tumour formation and progression. Therefore, 'Normalization' of the abnormal stromal environment should be able to impede or reverse tumour progression [186, 315].

Collectively, these observations indicate that the tumour microenvironment is a potential therapeutic target. The genetic stability of stromal cells as compared to the cancer cells can be exploited, as the therapies directly against the stroma are less likely to develop resistance. Treatment with non-steroidal anti-inflammatory drugs (NSAIDS) inhibits the

inflammatory cells and cytokines thus reducing the risk of colon and breast cancer and might prevent lung, esophageal and stomach cancer [316]. One of the best-studied and successful ways to normalize the stroma is to block VEGF signaling. Drugs designed to block VEGF are proved to be successful in the treatment of colorectal cancer. Furthermore, bevacizumab (sold under the trade name Avastin) – a VEGF blocking monoclonal antibody prolonged the life of colorectal cancer patients in combination with first-line 5-flurouracil chemotherapy in phase III trials [317]. Following this, FDA approved Avastin in February 2004 for the treatment metastatic colorectal cancer [318]. Inhibiting the signaling via EGFR with a neutralizing antibody (Transtuzumab) down regulates the tumour cell derived pro-angiogenic molecules. Transtuzumab impedes the proliferation of EGFR-expressing cancer cell and also exerts this effect on stromal cells which leads to slow tumour growth in patients with breast cancer. Transtuzumab (sold under the brand name Herceptin) is FDA approved in September 1998 for treatment of HER2-positive breast cancers [319, 320].

A delicate balance exists between the tumour-inhibitory and tumour-promoting properties of stromal cells, which are a limitation of targeting tumour environment. Hence, the key molecular differences between stromal cells under normal homeostasis versus when they have been co-opted or altered have to be identified and specifically targeted. A list of compounds targeting the tumour microenvironment is summarized in table 3.

Table 3: List of compounds / inhibitors targeting the tumour microenvironment in cancer (Adapted from [186])

Target	Approach	Clinical trial	Outcome/Status
Endothelial	Inhibition of VEGF signaling	Phase III for colon carcinoma, lung carcinoma and rental-cell carcinoma; Phase II for lung carcinoma and rental-cell carcinoma	Improved survival for colon carcinoma; Phase II studies for lung and rental-cell carcinoma show slightly improved survival and Phase III trials for these are ongoing
	Inhibition of endothelial-cell proliferation by TNP 470, a fumagillin analogue	Phase I/II for lung carcinoma and advance solid tumors	Drug well tolerated: some patients with partial responses
	Induction of apoptosis in proliferating endothelial cells with tubulin-binding agents	Phase I for thyroid cancer	Ongoing
Inflammatory cells	NSAIDs	Phase II for colon carcinoma	Ongoing with first beneficial results

ECM components	Local injection of radiolabeled antibodies against tenascin	Phase I and II for glioma	Increased survival
ECM/basement-membrane signaling	Antibodies against integrin	Phase I and II for lymphoma, melanoma and glioblastoma	Ongoing
ECM integrity	MMP inhibitors	Phase I, II, III	Initial results were negative; new components and combinations are in Phase I
ECM fragments	Injection of endostatin	Phase I	Ongoing

Targeting tumour microenvironment in the brain: Challenges: With regard to the brain tumours, targeting the tumour microenvironment possesses additional challenges due to the presence of blood-brain barrier (BBB). Pericytes surrounding the brain vessels, astrocyte end feet processes and smooth-muscle cells constitute the BBB. These organized cells contribute to the tightness of BBB and prominent tight junctions between brain endothelial cells and metabolic barriers, thus restricting the passage of cells and even small molecules through the BBB [321].

Upon, the initiation of brain metastases or primary brain tumours, in smaller malignant lesions, the BBB remains intact. In larger lesions, the vessels become leaky and the pericytes and astrocytes dissociate form the vessel wall. The BBB is affected to a variable degree within and between different primary and metastatic brain tumours [322]. Despite the increased leakiness in the tumour vessels as compared to the normal brain vessels, the uptake of chemotherapeutic drugs was lower than 15% of that found in the other tissues [323]. In addition, BBB permeability is regulated by pericytes by controlling the cerebral blood flow and clearance of toxic cellular byproducts. Tumour vessels vary in pericyte coverage. Tumour vasculature is better protected that the naked regions of brain endothelium [324]. In all, BBB pose a major challenge to be combated for an effective targeting of brain tumour microenvironment and drug delivery to the brain tumour site.

Targeting brain tumour microenvironment: Therapeutic opportunities

Targeting angiogenesis: Primary and metastatic brain tumours express high levels of VEGF [325]. This resulted in considerable efforts to target brain tumour growth with anti-angiogenic therapies. Phase II clinical trial with cediranib (VEGF tyrosine kinase inhibitor) lead to a rapid and prolonged 'normalization' of the tumour stroma in patients with recurrent glioblastoma [326]. In line with the above, treatment with bevacizumab also resulted in enhanced survival and normalization of tumour blood vessels. Furthermore, anti-VEGF therapy coupled with cytotoxic therapy reduced the BTSC population in orthotopic

brain tumour models [327, 328]. Disappointingly, anti-angiogenic therapies have also reported to sustain progression of primary and metastatic brain tumours via the cooption of pre-existing blood vessels in the adjoining healthy brain parenchyma [329, 330].

Indirect targeting of brain tumour microenvironment: The diversity of the cells present in the tumour microenvironment of the brain could be exploited as cellular vehicles to deliver therapeutic agents to the brain tumours and to normalize the tumour stroma. Genetically engineered Tie2 expressing monocytes expressing interferon-α (IFN-α) up-regulated IFN-inducible genes in the host compartment and inhibited angiogenesis. Due to the specific homing of Tie-2 expressing monocytes to the tumour site, no systemic toxicities were observed [331]. Similarly, neural stem cells and mesenchymal stem cells tagged with therapeutic agents also successfully inhibited brain tumour growth in animal models [332-334].

Other potential targets in brain tumour microenvironment: Microglia / macrophages may either have tumour-promoting or inhibiting characteristics. Therefore, it is possible that different populations of macrophages play distinct roles in brain tumours. A further study on the different sub-populations of these macrophages and ways to tune them to regress brain tumour is necessary [264]. Astrocytes may promote growth and chemotherapy resistance in brain tumours. Although, astrocytes cannot be directly depleted, individual molecular pathways responsible for tumour progression in astrocytes may be targeted. Interactions between pericytes and is crucial for intracranial tumour growth, which makes pericytes as lucrative therapeutic targets. Similarly, inhibiting the recruitment of endothelial cells to the tumour site and depleting the BTSCs could also hamper brain tumorigenesis [264].

1.5 Cell Motility and cancer

Cell migration is a central process in the development and maintenance of multicellular organisms. The orchestrated dislocation of cells in particular directions to specific locations is required for tissue formation during embryonic development, wound healing and immune responses. In the context of cancer, cancer cells undergo migration and invasion, which allows them to change position within the various tissues in the human body.

1.5.1 Mechanisms of cell motility

The process of cell migration is the fundamental machinery that allows the neoplastic cells to enter lymphatic and blood vessels for dissemination into circulation and then undergo metastatic growth in distant organs [335, 336]. To migrate, the cell body must modify its shape and stiffness to interact with the surrounding tissue structures. Hereby, the ECM functions as a substrate as well as a barrier towards the advancing cell body [337]. Cell migration through tissues results from a continuous cycle of 6 interdependent steps as listed below:

1. Cell polarization
2. Formation of protrusions at the leading edge of the cell

3. Formation of focal contact
4. Focalized proteolysis
5. Actomyosin contraction
6. Detachment of the trailing edge

Cell polarization: Cell polarization refers to the tendency of a migrating cell to have a distinct, stable front and rear end. The polarity is reinforced and it often arises from tumour microenvironment that provides directional cues. These directional cues can be chemotactic, (induced by growth factors, cytokines, chemokines), haptotactic (caused by varying concentrations of substrate) and / or mechanotactic (breakdown of cell-cell contacts, as in wound healing) [338]. There are various different proximal signaling pathways by which the cell is polarized and regulated, and it often varies between various cancer cell types and the tumour microenvironment. The establishment and maintenance of polarity during directional migration is mediated by signaling pathways involving integrins, phosphoinositides (PtdIns), cytoplasmic adaptor proteins and Rho family GTPases [203]. Generation of a haptotactic environment is necessary for efficient directional movement. This is achieved by the asymmetric recruitment of PtdIns and its regulator PtdIns-3-OH kinase (PI3K) to the leading edge, where it participates in a positive feedback loop with cdc42 and Rac to generate persistent polarization and directed migration [339]. Directed migration can also be regulated by PI3K independent mechanism involving Phosolipase C gamma (PLC-γ) pathways and integrin-paxillin and G1T1 cascade [340]. Central to polarity regulation are the Rho GTPases cdc42 and Rac1, which are active at the leading edge of polarized cells [341].

Apart from cell polarization, some tumour may require a polarized microenvironment for effective migration and invasion. Collagen fibers surrounding the normal epithelial structure and lungs are typically curly and anisotropic. However, following tumour initiation, collagen fibers at the vicinity of tumour microenvironment progressively thicken and linearize [342]. Linearized fibers are stiffer than curly ones and can substantially potentiate tumour cell migration. Thus cancer cells exploit this remodeled 'linear' collagen as invasion 'highways' [343, 344].

Formation of protrusions at the leading edge of the cell: Protrusion is a de nova formation of membrane extensions at the leading edge (*i.e.* in the direction of migration) of the migrating cell [345, 346]. It involves three consecutive steps:

1) The expansion of the plasma membrane and the formation of an underlying backbone that supports membrane extensions
2) The establishment of contacts with the substratum (ECM), which provides traction for the movement of the rest of the cell body
3) The activation of signaling cascade that regulate actin polymerization

Local actin polymerization is a key step in the formation of protrusions. The polymerized actin filaments form the backbone of a protrusion. Actin filaments adopt different morphologies depending on the number of filaments and the type and number of actin-

binding proteins that associate with the filaments. The results of these associations are filament bundles or a branched, dendritic structure. Further detailing of these associations lead to different cellular structures such as filopodia, lamellipodia, stress fibers or arches (dorsal or ventral) [347]. The specifics of these cellular structures are as follows (Figure 7):

Filopodia: They are made of long, unbranched, parallel actin bundles often decorated with tropomyosin, fasin and myosinX. They emerge as long, thin protrusions and their elongation is mediated by formins. Filopodia, mostly regulated by the Rho GTPases, have an exploratory function, enabling the cell to probe its local environments [348].

Lamellipodia: In contrast to filopodia, lamellipodia are broad, sheet like protrusions that contain a branched network of thin, short actin filaments. Rac GTPase generates Lamellipodia; whose downstream effectors are WAVE/Scr or WAVE-N, which regulates the Arp2/3 (actin nucleation complex) [349]. Lamellum is a structure often found behind the lamellipodium in which actin is bundled rather than branched. Most motile protrusions display a thin (≈1µm) lamellipodium close to the membrane, succeeded by a wider lamellum (5-10µm) closer to the nucleus [350].

Stress fibers: Often found along the ventral portion of the cell, stress fibers are thick bundles of actin filaments that contain many anti-parallel actin filaments. Stress fibers terminate in large adhesive structures known as focal adhesions at the end of the cell [351]. These structures are heavily decorated with myosin II, which grants them with contractile properties. The small Rho GTPase RhoA is a key regulator of stress fiber formation [352].

Arches (dorsal or ventral): Arches provide transverse structural support for the cell and are made up of long, thick bundles of actin filaments. They are bound to the dorsal and side portions of the cell and sometimes terminate in adhesions [351, 353].

The formation of the protrusions principally depends the 2D or 3D migratory environment. In 2D environments, cells generally form filopodia and invadopodia. Filopodia are transient and extend out from the lamellipodium, whereas invadopodia are more stable structures with substrate degradation properties that localize beneath the cell body. In 3D environments, filopodia-like protrusions comparable to filopodia or invadopodia described in 2D are formed. Depending upon the cell type, these filopodia like protrusions can also be referred as actin spikes or invadopodia like structures [347].

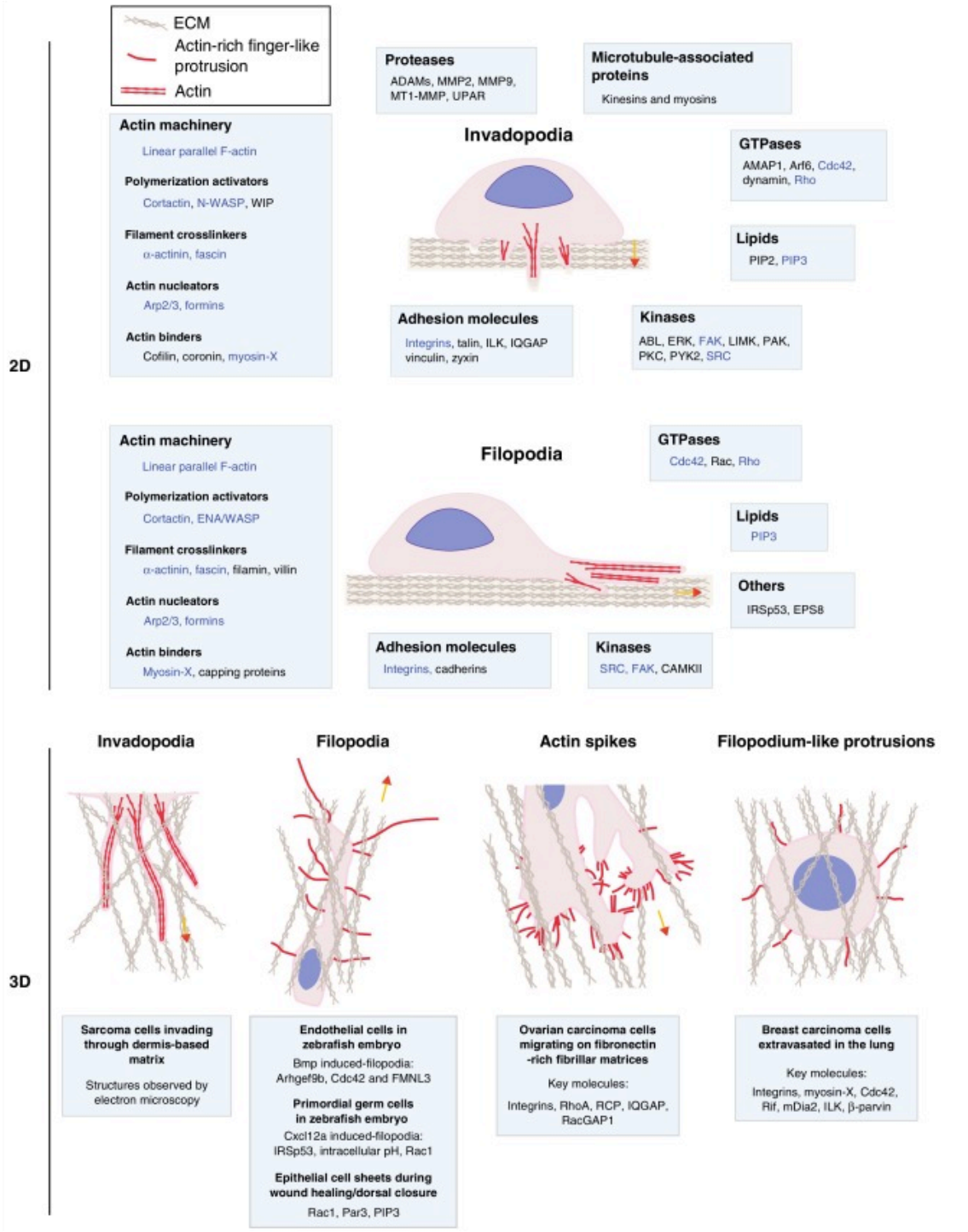


Figure 7: Key molecules involved in the actin dynamics of a migrating cell (Top). Schematic representation of different types of cellular protrusions for cell migration / invasion in 3D environments as adapted by different tumour and stromal cells (Bottom). (Adapted from [347]).

Actin polymerization is a multistep, highly regulated process, which results from the nucleation of new filaments and addition of GTP-bound G-actin monomers to the barbed (+) end of F-actin. Profilin and thymosin β4 are two molecules that regulate the availability of actin monomers for polymerization [354]. Both molecules bind specifically to G-actin. Profilin functions as an actin monomer 'shuttle' by binding to different actin polymerization nucleators and stimulators (e.g. formins) while thymosin β4 does bind to any nucleator

[355]. The latter sequesters G-actin and maintains an appropriate reservoir that enables the release of G-actin to profilin, to promote the growth of actin filaments. The pool of monomeric G-actin is maintained and derived from *de novo* synthesis (actin nucleation at pointed and barbed ends) and recycling of preformed filamentous structures by depolymerization at the pointed (-) end [345, 356].

Barbed end polymerization is more common and much more efficient than the pointed end polymerization. Several proteins, which can be divided into two antagonistic groups, regulate this process: barbed-end polymerization promoters and capping proteins. One example of barbed end polymerization promoters is the formins. Formins maintain filament elongation by remaining bond to the barbed end, simultaneously preventing binding of capping proteins [357]. mDia1, mDia2 and mDia3 are the best characterized formins and their activity is regulated by binding of small Rho GTPases (RhoA regulates mDia1 and cdc42 regulates mDia2) [358]. Other proteins, such as vasodilator stimulated phosphoprotein (VASP) and its relatives Mena and Ena/VASP-like (EVL) also prevent binding of capping proteins and promote actin polymerization via profilin [359, 360]. Capping proteins terminate elongation by binding to the barbed ends with high affinity in the presence of calcium, thereby limiting polymerization of existing filaments. The best known capping protein is gelsolin / brevin which is one of a series of family members that include capG, severin, villin, adseverin, advillin and supervillin [361, 362].

Branched actin polymerization complex is mainly carried out by the actin-related proteins 2 and 3 (the Arp2/3 complex). The Arp 2/3 complex is a heteroheptamer that binds to the side of a preformed actin filament and promotes actin polymerization, forming a 70° angle with the pre-existing filament [363]. This binding and subsequent polymerization imposes a branched geometry on the actin network. The Arp2/3 complex is activated locally at the cell membrane by phosphorylation of the proteins of the WASP/WAVE family [364]. The WASP family consists of 2 main members: Wiskott-Aldrich syndrome protein (WASP) and neuronal-WASP (WASP/N). WASP/N-WASP/WAVE regulation of Arp2/3 complex is tightly regulated by small Rho GTPases [365].

Depolymerization is a spontaneous process that occurs at the pointed ends owing to filament ageing or to filament severing catalyzed by cofilin, an ADP depolymerization factor. As a consequence of its severing function, cofilin functions dichotomously by creating new free barbed ends from which polymerization can be reinitiated and also breaking down and depolymerizing old networks for monomer recycling [366]. Cofilin is regulated by phosphorylation of LIM kinase (LIMK), which acts downstream of p21-activated kinase (PAK) or Rho-kinase (ROCK) signaling cascade [367].

Formation of focal contact: Specific adhesions to the ECM as required by the invading cell occur mainly via integrin receptors, which are linked to the F-actin cytoskeleton through talin, vinculin and α-actinin [368]. Integrin heterodimerization triggers signaling pathways that link the substratum to the actin cytoskeleton and thereby provides traction for migration. These sites of adhesion are usually spatially restricted and vary from small and dot-like (nascent adhesions or focal complexes) to large and elongated structures (focal

adhesions). The shape, size and functional role of the adhesions vary with their subcellular localization and cell type [368]. Phosphorylation of focal adhesion kinases (FAK) is rapid event that is associated with the formation of focal contacts. FAK is a cytosolic protein tyrosine kinase (PTK) and a critical mediator of cell adhesion signaling between ECM and focal adhesions via integrin. Unlike many other cytosolic PTKs, FAK does not contain an SH2 (src-homology 2) or SH3 (src-homology 3) domain [369, 370]. Several sites of FAK tyrosine phosphorylation have been identified in FAK, which serve to modulate FAK kinase activity and various downstream signaling pathways that are implicated in cell migration. One particularly well-characterized pathway is through the association and phosphorylation of p130cas by the FAK/Src complex [371-373]. Tyrosine phosphorylated p130cas associates with several SH2-containing proteins like Crk. The Cas/Crk complex further transfers the signal downstream via DOCK180 and Rac, which regulate membrane ruffling and focal adhesions [374].

Paxillin is also a major substrate of the FAK/Scr kinase complex and recruits Crk to the cell migration cascades similar to p130cas. Paxillin is also found in a multi-protein complex containing another adaptor molecule PKL, a guanine nucleotide exchange factor Pix/Cool whose phosphorylation is also dependent on FAK. The phosphorylation of Pix/Cool-FAK complex is also attributed by cdc42/Rac target-effector PAK that may link its downstream targets LIMK and MLCK to regulate cell migration [375]. FAK also regulates cell migration through its effectors of the Rho superfamily of small GTPases by the assembly and disassembly of actin cytoskeleton components [376].

Focalized proteolysis: An effective strategy to remodel the ECM to overcome spatial restrictions or to provide the appropriate ambience for the moving cell is by removal of one or more of its components. Virtually all protein components outside or inside a cell are subject to degradation and modification [377]. The most substantial enzymes in ECM remodeling are metalloproteinases. Matrix metalloproteinases (MMP) and a disintegrin and metalloproteinase with thrombospondin motifs (ADAMTS) are two families of metalloproteinases that are specialized in degrading the ECM. Serine proteinases, which include plasmin and cathepsin G degrade ECM protein components extracellularly at a neutral pH. In contrast, cysteine, aspartate and threonine proteinases are predominantly active at acidic pH and mainly digest intracellular proteins [378].

There are 23 known members of MMP family in vertebrates. All MMP have a basic three-domain structure with self-inhibitory prodomain at the amino terminus, a catalytic domain, a flexible hinge domain and a hemopexin domain at the carboxyl terminus. However, certain MMP harbor variations to facilitate the degradation of specific ECM components [379]. MMP-2 and MMP-9 have fibronectin type II repeats inserted in the catalytic domain to mediate its binding to collagen. MMP target a wide range of ECM and other extracellular proteins. MMP-3 and MMP-10 specifically target proteoglycans; fibronectin and laminin while MMP-2 and MMP-9 degrade denatured collagen (gelatin). MMP-1 prefers collagen III, MMP-8 and -13 selectively digest collagen I and II respectively [380, 381].

Like MMPs, ADAMTS proteinases are multigene family, consisting of 19 members of closely related metalloproteinases. ADAMTS have a thromobospondin type-1 (TSP-1) repeat, cys domain and one or more additional TSP-1 repeats [382]. ADAMTS are aptly called as the 'proteoglycanases' as they are effectual for degradation of ECM proteoglycans. Indeed, ADAMTS-1, -4, -5, -8, -9, -15, -16 and -18 degrade aggrecan, versican, brevican and other proteoglycans. In contrast, ADAMTS-2 participates in the removal of amino prodomain from the procollagen I in the dermis [383].

The serine protease plasmin degrades matrix proteins such as fibronectin, fibrin and laminin, and thus contributes to ECM remodeling. Likewise, neutrophil elastase and cathepsin G are also involed in ECM remodeling and degradation [384]. The extracellular sulfatases SULF-1 and SULF-2 are extracellular enzymes that remove 6-*O*-sulphates from heparin sulfate proteoglycans alerting Wnt, VEGF, PDGF, FGF and other signaling events [385].

Actomyosin contraction: Before and during the development of focal adhesions, actin filaments locally elongate and assemble, through the action of cross-linking proteins such as actinin and myosin II. Actin reorganization, rather than polymerization, is the leading mechanism thought to create the architecture in the invading cell [386]. Reorganized actin filaments are organized and bundled together in thick, linear, and mostly anti-parallel arrays. Branched actin networks termed, as 'cortical actin' are present below the inner leaflet of the plasma membrane, whereas elongated cables of actin filaments designated as 'stress fibers' are localized in the cytoplasm [387]. The contraction of actin filaments in an invading cell is provided by myosin II (in non-muscle cells) by moving directionally along actin filaments or polarized actin bundles. These stresses are important for cellular retraction of the moving cell. Myosin II generally forms 'minifilaments'-bipolar polymers of tens of molecules with active heads at both ends of actin. This active heads move towards the barbed-ends and away from the pointed ends. These ordered actin-myosin arrangement allows for a straightforward contraction in the cells [388]. Stress-fiber assembly and contraction, which are controlled by myosin II are predominantly, induced by the small G-protein Rho and its downstream effector ROCK. In contrast, the cortical actin network is regulated by the myosin light-chain kinase (MLCK), but not by RHO [389]. This clear segregation of control mechanism allows the cell to control cortical actin dynamics and contractility separately. Actomyosin contraction promotes the shortening of the cell's length axis and generates inward tension towards focal adhesions that are located at the outward edges [390].

Detachment of trailing edge: Cell body translocation propelled by a coordinated contraction of the actomyosin cytoskeleton is not well understood. Cellular translocation is controlled in part by myosin II and microtubule motors (e.g. dynein). Rear end retraction requires the coordinated contraction of the actin cytoskeleton and disassembly of the adhesions at the trailing edge [391]. Multiple mechanisms congregate to promote focal adhesion contacts disassembly: actomyosin contraction that exerts force against the adhesion promotes its detachment (many cell types leave tracks of integrin receptors behind), microtubule-induced focal adhesion disassembly, integrin endocytosis and proteolytic cleavage by calpain of focal adhesion proteins that link the integrins to the actin. Following the focal adhesion

disassembly, the cell body and nucleus slowly glide forward and pull the trailing edge forward [392, 393].

1.5.2 Modes of cancer cell motility

A given cell type might preferentially use one particular adhesion and migration mechanisms. The various migration preferences of the tumour cell is modulated by the growth factors / cytokines and matrix components in the tumour microenvironment. Motility-inducing chemokines and growth factors induce and maintain migration by pro-migratory signal transduction cascades [215]. Furthermore, these pro-migratory signaling cascades determine how a migrating cancer cells move through tissues and become organized into invasive tumours.

Extensive in-*vitro* and *in vivo* observations have shown that tumour cells infiltrate neighboring tissue matrices in diverse patterns [394]. Tumour cells can disseminate as single, individual cells referred to as 'single cell migration' or infiltrate as a group as cell strands, sheets, files or clusters called as 'collective migration' (Figure 8). Simultaneous presence of single cell and collective migration is possible in many tumours [395, 396]. Leukemia, lymphomas and solid stromal derived tumour cells from sarcomas disseminate via single cell migration whereas epithelial tumours commonly exploit collective migration mechanisms. The choice between single cell and collective migration depends on the differentiation stage of the tumour. The lower the differentiation stage, the more likely the tumour is to disperse via single cell migration [397]. These differences in cell migration likely reflect variations in the molecular repertoire used by a specific type of cancer cell to migrate.

1.5.2.1 Single cell migration

It is a well-documented phenomenon that individual tumour cells are motile, which is evident from the extensive *in vitro* and *in vivo* studies. Individual motile tumour cells usually originate from the interstitial stroma or bone marrow. Alternatively, cells that lose their local focal contacts from a multicellular compartment such as epithelium can also detach and migrate as individual cells through the adjacent tissues [398]. Based on cell type, integrin engagement, actin cytoskeleton structure and protease production, single cell migration can occur in different morphological variants. While there are multiple morphological variants, the most common ones are mesenchymal motility and amoeboid motility.

Mesenchymal motility: Mesenchymal cells move in the classical complete five-step migration cycle (as described in 1.5.1 mechanisms of (cancer) cell motility). Mesenchymal motility represents an efficient mechanism for tumour-cell dissemination and metastasis. In 3D tissues, mesenchymal cells adopt a spindle-shaped, fibroblast-like morphology, as characteristic for fibroblasts, myoblasts, single endothelial cells and sarcoma cells [399]. This unique elongated morphology is dependent on integrin-mediated focal adhesion dynamics and the presence of high traction forces at both the leading and rear edges of the cell [400].

The dependence on integrin is evident in fibroblasts, endothelial cells or tumour cells treated with integrin small-molecule inhibitor or blocking antibodies, which lead to cell retraction, acquisition of spherical shape and impaired migration rates [206]. Concomitant to the integrin and actin at substrate binding sites, proteases are indispensible prerequisites for meschymal motility. Mesenchymal cells recruit β1 and β3 integrin and surface proteases such as MMPs to digest and remodel the ECM. The proteases then co-localize at contact regions to ECM fibers and generate structural matrix defects along the cell migration tracks by proteolysis of the ECM near to the moving cell surface [401, 402]. Mesenchymal motility is a relatively slow mode of migration with focal contact formation and turnover occur in the timescale of 10-20 minutes, resulting in velocities (0.1-2μm/min) in 3D models [403, 404].

Small GTPases Rac and cdc42 generate pseudopod and lamellipod dynamics at outward edge of the migrating cells and its activity levels could be used as a marker for mesenchymal motility. Rac and cdc42 causes rapid and dynamic type of β1 integrin activation favoring engagement towards 2D and 3D substrata [405]. Interfering with Rac and cdc42 activity perturbs cell protrusions and polarized force generation, thereby severely impairing migration. In mesenchymal cells, active Rho leads to increased adhesiveness, stress fiber formation, disruption of elongated cell morphology and retardation of migration speed [406, 407]. Together, Rac and cdc42 mediate the coordination and synergy between polarized cytoskeletal dynamics at the leading edge of a mesenchymal cell while it is opposed by Rho. Although Rho exerts an opposing effect on mesenchymal motility, Rho-mediated adhesion-strengthening and cell contractility are nevertheless important for migration and tail retraction of mesenchymal cells.

Amoeboid motility: Many established tumour cell lines when cultured in the 2D environment do not follow the characteristics of mesenchymal dynamics (due to the absence of ECM in 2D), but use a less adhesive amoeboid type of migration. Arguably the most primitive form cell migration is amoeboid movement, which mimics features of the single-cell behavior of the amoeba *Dictyostelium discoideum* [408]. *Dictyostelium discoideum* has been used a model organism to establish the characteristics of amoeboid motility. *Dictyostelium* is an ellipsoid cell that can rapidly deform (within seconds) and translocate via fast alternating cycles of morphological expansions and contractions [409]. Although integrins are not expressed and the binding force towards the substrate is relatively low, *Dictyostelium* cells utilize one or more non-integrin pattern recognition receptors like sadA to bind to the extracellular structures [410].

In higher eukaryotes, hemotopoietic stem cells, leukocytes, macrophages and certain tumour cells exhibit amoeboid movement. These cells habit a fast 'crawling' type of movement that is driven by short-lived and weak interaction with the substrate. In lymphocytes and neutrophils, β1-integrin-mediated adhesion is completely or partially replaceable for cell migration within the connective tissue [411]. Highly deformable leukocytes adapt amoeboid movement and move at high velocities (2-30μm/min) because of the lack of stable focal contacts [412]. Deformation and rapid morphological change is generated by cortical filamentous actin. T lymphocytes and other leukocytes use protease-independent physical mechanisms to overcome ECM barriers, including adaptation of the

cell shape to preformed matrix structures (contact guidance), extension of lateral footholds ('elbowing') and squeezing through narrow spaces (constriction rings). These shape-driven adaptations allow the amoeboid cells to glide through or circumnavigate the ECM barriers rather than to degrade them. The ellipsoid cell shape and the various shape-driven adaptations require a stiff and contracted cell cortex, which is facilitated by actin polymerization along the plasma membrane [413-415]. The small GTPase RhoA and its effector ROCK critically control the cortical actin dynamics. Activated ROCK in the invading cells generates cortical tension, stiffness and maintains the roundish cell morphology. While Rac and cdc42 are mostly active in mesenchymal motility, they can also generate dynamic cell protrusions, such as pseudopodia and blebs that support amoeboid movement [416]. In contrast to the five-step migration paradigm, focal adhesion contacts and focalization of proteolysis are thus eliminated in amoeboid movement, whereas fast and non-integrin receptor assemblies at the cell-matrix interactions are retained.

Other unusual modes of motility:

Chain migration: Chain migration occurs in non-neoplastic neural crest cells, myoblasts and melanomas. This mode of motility involves the formation of cell 'streams' one after another in a strand-like fashion [417, 418]. When cells form 'streams' and move in a chain, they form cell-cell contacts at the leading edge, indicating that some cell-cell adhesion and communications similar to mesenchymal motility is preserved. The cell 'streams' are characteristic histological features of epithelial neoplasias, metaplastic breast carcinoma, ovarian cancer and vascular-type melanoma [419, 420]. This arrangement of invading tumour cells in chains represents a peculiar effective penetration mechanism that confers high metastatic capabilities and poor prognosis [421, 422].

Lobopodial migration: Cells, which have adopted mesenchymal motility migrate by forming lamellipodia can form different migratory structures termed 'lobopodia' at the leading edge in some 3D environments. The cylindrical shaped lobopodia have features of both blebs and lamellipods, and are a type of protrusions in mesenchymal cells migrating in physiological 3D environments (Figure 9B) [423]. Unlike lamellipodia, these lobopodia accumulated neither active Rac nor cdc42 but the cells still formed focalized adhesions. These blunt-ended lobopods are driven by RhoA-dependent myosin II activity. Similar to blebbing cells, lobopodia were very sensitive to perturbations of actomyosin contractions. Decreased contractions instantly cause the lobopodia-adopted cells to switch to the classical lamellipodial migration mode [424, 425].

1.5.2.2 Collective migration

In collective migration cells move as multicellular connected strands or chords into tissues by maintaining their cell-cell junctions. In contrast to single cell migration, a special form of cortical actin filament assembly along cell junctions are formed by the cell-cell adhesions that occur among the cell groups. The so-called 'path-generating cells', a subset of mobile cells present at the periphery of the moving cluster generates migratory traction via pseudopod activity. Cells in the inner trailing regions are passively dragged behind the

moving cluster [426]. Cells at the leading edge of the cluster engage and cluster β1 integrins in anterior protrusions towards the ECM and actively degrade the ECM via MT1-MMP and MMP-2 mediated proteolysis. Sensitivity of collective clusters to integrin and protease antagonists proves the dependency collective migration on integrins and proteolysis [396, 427].

Two morphological and functional variants of collective migration have been identified in tumours as described below:

- Protruding sheets and strands that maintain contact with the primary tumour, yet display local invasion. This functional variant is observed in invasive epithelial cancer such as oral squamous cell carcinoma and mammary carcinoma, colon carcinoma and basal cell carcinoma [428-430].
- Detached cell clusters, histologically seen as 'nests' detach from their origin and extent along perineural structures. This variant is seen colorectal cancer [429].

Homotypic cell-cell interactions within multiple strands and sheets are regulated via cadherins (E-, N-, P-, VE- and cadherin 11), members of the immunoglobulin superfamily (e.g. ALCAM) and connexins, which are involved in communication through gap junctions [431, 432].

Collective cell invasion is a preferred mode of migration in highly differentiated tumours such as lobular breast cancer, epithelial prostate cancer and large-cell lung cancer as tissue infiltration by individual cells is rarely detectable in these tumour types. So, collective-cell movement could be a primary mechanism for invasion and metastasis in highly differentiated tumours. Collective cell migration confers a huge advantage to the invading tumour mass as large cell mass can produce high autocrine concentrations of promigratory factors and MMPs [433]. The inner cell mass is protected from immnuo-surveillance excreted by lymphocytes and natural-killer cells. In collective migration, cells of different clonal origin and with different biological properties can function together. The overall tumour invasion efficiency and survival probability is increased as more migratory cells can promote the invasion of less motile and apoptosis-resistance cells [434].

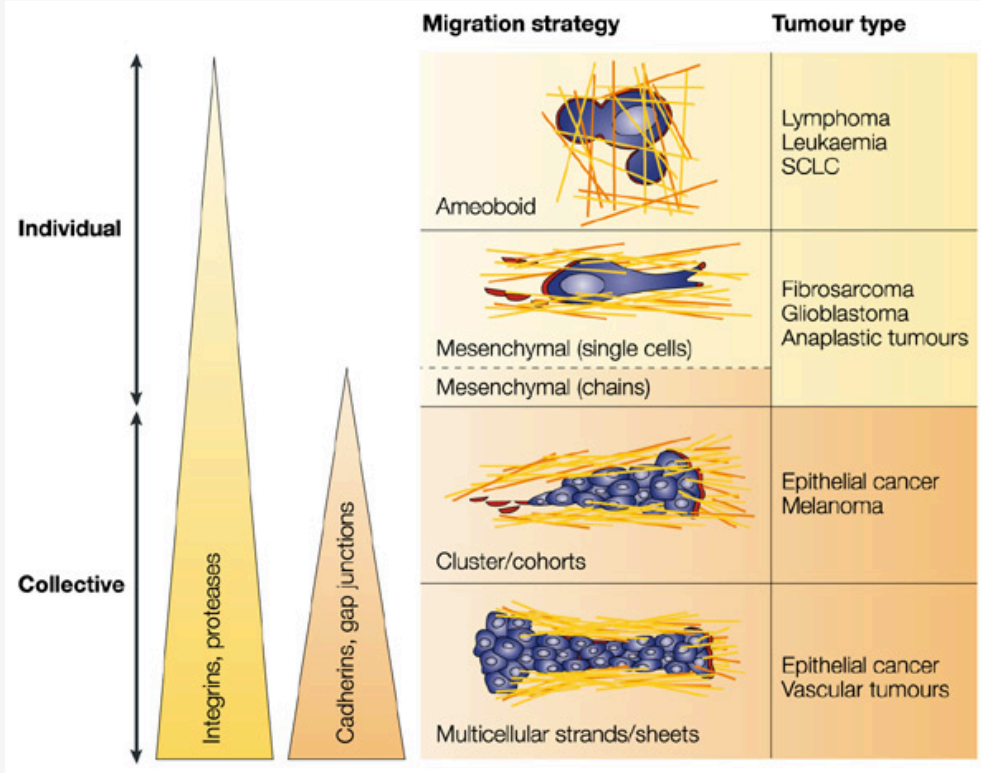


Figure 8: Different modes of cell motility: Individual single cell or collective cell migration strategies are determined by different adhesion molecules, proteases and gap junctions (Triangles). Schematic representation from individual (top) to collective (bottom) modes of migration as adapted by different cancer cell types. (Adapted from [434]).

1.5.3 Plasticity in modes of cell migration

Differentiated cells generally retain their mode of migration once they have acquired it, while in cancer cells, gain or loss of generically pre-specified components of the migration cycle can cause an adaptive switch between the different modes of migration. This phenomenon is termed as 'plasticity or transition' [394] and controlled by cell-cell adhesion, expression and function of integrins, traction force and ECM composition (Figure 9A, 9B) [435].

Epithelial – mesenchymal transition: The most well established example of transitions in cancer cell pattern and function is the epithelial-mesenchymal transition (EMT). During progressive de-differentiation in epithelial cancer, the cancer cells undergo a transition from a collective invasion to mesenchymal single cell migration termed as EMT [397]. The prerequisite for EMT is the loss of cell-cell junctions which is achieved by any of the following mechanisms listed below:

- Loss-of function mutations in cadherins [436].
- Up-regulation of proteases that cleave cadherins [435].
- Production of specific cytokines by the tumour microenvironment. Specifically, HGF downregulates cadherins and activates pro-migratory GTPases [437].

EMT is considered to be a significant step in the cancer invasion cascade, as once the tumour has achieved the dedifferentiated stage of single cells; the probability of cancer cell dissemination and metastasis is increased.

Mesenchymal – amoeboid transition: If mechanical or signaling pathways that stabilize the fundamental cell-ECM interactions required for mesenchymal movement are weakened, cancer cells can convert towards amoeboid migration [438]. There are three known mechanisms leading to mesenchymal-amoeboid transition (MAT).

I. Loss of protease function: Tumour cells that have adopted mesenchymal migration cease their proteolytic migration after the treatment with protease inhibitors that target MMPs, ADAMs, cathepsins and serine/threonine proteases [439-442]. The hold on migration was however temporary, as the cells rapidly switch to amoeboid migration involving shape change and the ability to squeeze through narrow regions. Consistent with the hallmarks of amoeboid migration, there was a change in integrin expression and the filamentous actin adopted a diffuse cortical pattern [415].

II. Activation of ROCK: Active RhoA is required for diffuse cortical actin polymerization and cellular retraction [443]. Overexpression of constitutively active ROCK causes cortical contractions and cell rounding in cells, which originally adopted mesenchymal motility [407]. Active ROCK also converts the cells to protease independent migration type, reminiscent of amoeboid movement [444].

III. Downregulation of integrin function: Interfering with β1 integrin function reduces attachment forces without affecting the cell contractility prompts cell rounding and transition towards amoeboid movement [445, 446].

Collective-amoeboid transition: Analogues to EMT, if cell-cell and cell-ECM interactions are destabilized, there is a transition from collective cell migration to amoeboid single cell migration. In multicellular clusters of melanoma, inhibition of integrin β1 abrogated collective cell migration yet induced detachment of individual amoeboid cells [447, 448]. The detached cells adopted amoeboid shape and diffused β1 integrin distribution pattern similar to that seen in migrating lymphocytes [431].

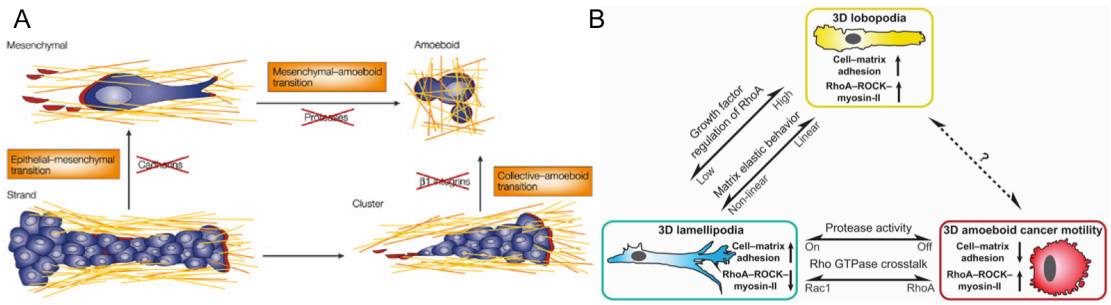


Figure 9: Plasticity in modes of motility: (A) Schematic representation of disseminating cancer cells, which can undergo a variety of adaptations / transitions in response to changes in their molecular migration program as modulated by the tumour microenvironment. (Adapted from [434]). **(B)** Mechanical control and transitions of the mode of 3D cell migration [423].

1.5.4 Importance of targeting cell motility in cancer

Ultimately, the aforementioned extrinsic factors and the underlying mechanisms determine the motility and metastatic ability of tumour cells. Successful targeting of cancer cell motility for therapeutic means lies in the identification of molecular mechanisms required for tumour cell motility. This offers several inroads for novel drug design and clinical intervention to limit cancer progression towards overt metastasis and to treat existing metastatic disease. Advantages of targeting cancer cell motility are as follows:

I. Cancer patients prone to development of systemic disease are treated aggressively. This scheme of treatment is frequently accompanied with high morbidity, poor quality of life and elevated levels of treatment related adverse effects. Instead, cancer cell motility could be inhibited as a preventive measure to enable patients to be kept under active surveillance without risking the appearance of systemic spread of cancer [449, 450].
II. Targeting tumour cell motility within the primary tumour could constrain local invasion. Surgical intervention is frequently ineffective in invasive neoplasia, like glioblastoma and pancreatic cancer as the disease often recurs due to aggressive local infiltration. These infiltrative neoplasias often prove to be incurable even in the absence of overt metastasis to distant organs due to the increase in tumour volume resulting from the local infiltration [450]. Therefore, targeting motility could improve therapy of these malignancies by counteracting further infiltration and expansion into the normal tissues.
III. Short-range dispersal of cancer cells could lead to the overall increase in the tumour volume and formation of small satellite tumours. These tumours are likely to be very heterogeneous from the primary tumours and are resistant to chemotherapeutics aimed for the primary tumour, thus leading to the rapid onset of resistance to chemotherapy [451]. Restraining cell motility could serve as an effective means to hamper local infiltration and eventually chemo-resistance.
IV. Dissemination of the cancer cells to the lymph nodes is the first step in a metastatic cascade. However, no improvement in survival is seen even after the removal of regional lymph nodes [452]. This suggests that cells responsible for metastases have not been mobilized from the primary tumour site and would therefore remain susceptible for anti-migration therapy.
V. Anti-migratory therapy could hinder the process of 'clonal evolution' towards an increasingly metastatic phenotype [453].

1.5.4 .1 Potential strategies to target cancer cell motility

Having stated the importance of targeting cancer cell motility, anti-migration therapy has a tremendous potential in the clinic. An ideal cell motility targeted therapy should limit, intervene and disturb the molecular processes that support the migratory pathology without affecting the normal physiological functions. Pro-migratory signals specifically up-regulated in cancers such as MMPs, tenascin-C, or common nodal points for the cell phenotype can be targeted to curb motility [450]. Targeting specific molecules that are over-expressed in

cancers confers limited toxicity. However, the aforesaid approaches are limited by the availability of other pro-migratory signals, indicating that abrogation of one would be a short-lived benefit.

An alternative approach would be to provide anti-motility signals rather than to restrict pro-migratory pathways. Up-regulation of physiological 'stop' signals such as CXCR3 and decorin could be triggered in a therapeutic manner to limit invasion. This approach has to be translated with caution, as tumour cells are capable of turning the 'stop' signal to 'go' signal via splice isoform switching [454]. Currently, no therapy targeting tumour cell motility has been approved for clinical use. The list of various anti-migrating therapies in clinical trials is comprehended in the table 4.

Table 4: List of anti-migratory therapies and their clinical trials. (Adapted from [450])

Drug	Target	Company	Clinical Phase
Cell autonomous			
Saracatinib (AZD0530)	Src	AstraZeneca	II
Bosutinib (SKI-606)	Src	Wyeth	II, III
Dasatinib (BMS-354825)	Src	Bristol-Myers Squibb	I, II
Fasudil	Rho kinase	Asahi Kasei	I, II, III
Emodin	Cdc42/Rac1		I
Soluble interactions			
CTCE-9908	SDF-1	Chemokine Therapeutics	I, II
MetMAB (PRO143966)	Met	Roche/Genentech	II
AMG 208	Met	Amgen	I
GC1008	TGF-β family	Genzyme	I, II
Trabedersen (AP 12009)	TGF-β2	Antisense Pharma	I, II
Infliximab	TNF-α	Centocor	I, II
EGFR	Herceptin (trastuzumab)	Genentech/Roche	I, II, III
VEGF	Avastin	Genentech/Roche	I, II, III
CXCR-4	CTCE-9908	Chemokine Therapeutics	I, II
Cell-cell interactions			
IGN-101	EpCAM	Aphton	I, II
Exherin (ADH-1)	N-cadherin	Adherex	I, II
Cell-matrix interactions			
Cilengitide (EMD121974)	αvβ and αvβ5 integrins	EMD/Merck KGaA	II, III
Volociximab (M200)	α5β1 integrin	PDL/Biogen Idec	II
Etaracizumab (Abegrin)	αvβ3 integrin	MedImmune	I, II
ATN-161	Integrins	Tactic Pharmaceuticals	I, II
BMS-275291	MMPs	Bristol-Myers Squibb	I, II, III
Endostatin	MMPs	Alchemgen	III

		Therapeutics	
Curcumin	MMPs	Sabinsa Corporation	I, II
Tigapotide (PCK3145)	MMP9	Ambrilia Biopharma	II
A6	CD44	Angstrom Pharmaceuticals	II
Mesupron (WX-671)	uPA	Wilex	I, II
ATN-658	uPA	Tactic Pharmaceuticals	I
Tempostatin (Halofuginone hydrobromide)	Stroma	Collgard Pharmaceuticals	II
PI-88	Heparanase	Progen	I, II, III
Vitaxin	αvβ3	MedImmune	II
Molecular integration			
CFAK-C4	FAK	CureFAKtor Pharmaceuticals	I
PF-562271	FAK	Pfizer	I

1.5.4.2 Challenges in targeting cell motility in cancer

Complete inhibition of pro-migratory pathways may achieve anti-migratory effect, but partial inhibition may be counterproductive. Actuation of migration depends on a balance of pro-migratory signaling pathways and any changes to this balance via incomplete inhibition may activate other compensatory pro-migratory signals. Compensations mechanisms like this may be a major limiting step during long-term maintenance therapies as opposed to short-term ablative therapies currently used to kill cancer cells [455]. Furthermore, there is a possibility that tumour cells have disseminated quite early prior to detection and initial treatments [456].

The principle obstacle to develop progression-targeting therapies is the design of clinical trials to determine the efficacy. Anti-metastatic therapies are tested as adjuvant therapies after the standard first line therapies. The standard measurement for efficacy involve shrinkage of tumour size / volume, but anti-migratory agents would require progression-free or overall survival as measurements as early as in phase II trials. Patients enrolled in such trials will have to be under continuous treatment because it is not possible to precisely predict when a cancer becomes metastatic. This treatment regimen will incur negative effects on immunity and wound healing. Consequently, all the drugs currently under investigation target not only motility but also influence cell viability and proliferation. Hence, it is difficult to distinguish the true target and effect of the anti-migratory drugs [449, 450].

1.6 Cell motility models

1.6.1 Current in vitro models to study cell motility

A number of *in vitro* methodologies have been developed to characterize cell locomotion more easily and thus identify novel anti-migratory drugs and targets on cell migration. *In vitro* test provide the initial range of information about cell migration and can generally be scaled-up to high throughput assays. This circumvents the limitations of *in vivo* methods, which are habitually expensive, time consuming and low throughput [457, 458]. A list of well established 2D and 3D cell migration / invasion assays are explained below:

Assays in the 2D environment: Cell migration assays:

Transwell migration assay (Boyden chamber assay): This assay is based on the cells ability to transmigrate a porous membrane, which separates tow medium containing chambers. In brief, the cells are seeded in the upper chamber and the cells migrate in a vertical direction through the pores of the membrane to the lower compartment, which contains attractants such as chemokine or growth factors [459]. **Quantification:** There are two methods to detect and quantify the migrated cells. The cells that transmigrated the membrane can be fixed on the membrane, stained with hemotoxylin, toluidine or crystal blue and the cell number can be counted. Alternatively, the migrated cells can be fluorescently stained, dissociated from the membrane and quantified using a fluorescent reader. Use of dark coloured porous membrane (FluoroBlok, Becton Dickinson) can block light transmission from non-migrated cells thus rendering easy quantification of the cells without dissociation. **Advantages**: Relatively easy set-up to asses the effects of growth factors / cytokines gradients and this assay is available for different cell culture inserts and sizes. 96-well multiwell transwell assay for high throughput screening of cell migration are recently developed (www.neuroprobe.com). **Disadvantages:** It is an endpoint assay; time-lapse analysis of the effect of attractants cannot be determined using this assay [458].

In vitro wound healing assay (Scratch assay): This is one most popular and commonly used 2D migration assays. The assay is based on scraping off (wounding) an area of cells in a confluent plate of any type of adherent cells which is normally done using a pipette tip. Cells migrate from the non-wounded areas into the wound, a process that can be monitored microscopically [460]. To add a layer of complexity to this assay, cells migration can be assessed on plates coated with ECM proteins (collagen, laminin, fibronectin or basement membrane extract (also called matrigel)) [461]. **Quantification:** The extent of cell migration (single cell or collective) can be measured in terms of decrease of the uncovered region at different time points until the 'wound' is closed. A freeware 'TScratch allows simple automated image analysis and quantification [462, 463]. **Advantages:** Simple, rapid set-up and cheap. **Disadvantages**: Long-term effects cannot be assessed, as the assay after 24-hour time point cannot distinguish cell proliferation and changes in cell survival from cell motility. Only adherent cells can be studied using this assay. In addition, the scratch is often uneven which has to be considered during quantification. Rather, electric cell-substrate impedance

sensing (ECIS) as developed by Applied biophysics (www.biophysics.com) can be used to create wounds of defined size [464].

Cell exclusion zone assay (Platypus migration assay): This assay was designed to overcome the problem of uneven wound in the scratch assay. Small silicone stoppers that fit into each well of a 96 well plate are positioned prior to cell seeding to create an exclusion zone devoid of cells. After cell adhesion, the stoppers are removed allowing the cells to migrate into the zone of exclusion. Recently developed derivative of this assay employs precast dissolvable biocompatible gels instead of silicone stoppers. **Quantification:** Migration is measured either by image analysis of area covered by the cells in a given time or using microplate reader if the cells are fluorescently labeled. **Advantages:** The assay is high-throughput (available in 96 well and 384 well format). The assay is standardized, reproducible and easy to set-up. **Disadvantages:** This assay is not suited for non-adherent cells. No growth factor / cytokine gradients can be formed; the effect of only single concentration of an attractant can be assessed [457].

Fence assay (ring assay): This assay is an inversion of cell exclusion zone assay. A ring (fence) made of Teflon, glass or metal is place on a standard cell culture dish and the cells are seeded inside the inner ring of the fence. Following the adherence of the cells, the ring is removed allowing the cells to migrate away from the confluent area [465]. Due to the same principles the methods of quantification, advantages and disadvantages are similar to the cell exclusion zone assay.

Capillary chamber migration assay (microfluidic chamber assays): This assay involves the use of two chambers placed in a horizontal setting side-by-side and separated by a narrow connecting bridge. One of the chambers is seeded with cells and the other chamber is filled with an attractant thus creating a stable gradient between the chambers [466]. **Quantification:** The number of migrating cells is counted on the surface of the connecting bridge using light microscopy. **Advantages:** This assay is often used for evaluating leukocyte migration. Only small volumes of re-suspended cells are required making this assay well suited for testing rare cells types and expensive compounds. Easy handling and the assessment of directed chemotaxis along a gradient is possible [467, 468]. **Disadvantages:** Automation processes are difficult to set up with the available systems.

Single cell migration assay (colloidal particle assay, colloidal gold single cell migration assay): Cells are seeded onto colloidal gold particle coated tissue culture dishes at low density ($1X10^3$ cells/ml). The gold colloidal particles appear as a layer of homogenous dark dots under the microscope. When the cells migrate, the gold particles are removed form the plate by the cells via phagocytosis. This results in white cleared tracks. **Quantification:** The tracks created by the migrating cells are imaged and the cleared areas can be analyzed quantitatively [469]. **Advantages:** This assay provides details of migrating cells at a single cell level. Undirected movement, real-time path detection is feasible thus allowing the absolute determination of speed / velocity of migration. **Disadvantages:** The assay is not high throughput and very labor intensive. Automated systems are needed to ease the use of this assay.

Time-lapse / cell tracking: With the advent of video microscopy, the individual movement of many cells can be analyzed at once and the cell migration paths articulate information about the total migration distance, direction and velocity at any given time point. The choice and tracking of cells can either be performed manually, semi-automated or fully automatic. Algorithms for automated cell tracking have been developed to aid with migration paths of several cells simultaneously and cope with cell division of moving cells [470, 471].

Applications of 2D migration assays:

- To analyze the influence of chemoattractants on migration and invasion [472].
- To study the influence of other cell types like macrophages and fibroblasts on invasion and migration of cancer cells [473].
- Isolation of invasive cells from non-invasive cells for molecular analysis [474].
- To test the influence of knockdown, transfection and antibody treatment on migration [475].
- To assess drug therapies in reducing invasion [476].
- To assess the role of soluble factors like calcium on invasion [477].

Assays in the 3D environment: Cell invasion assays:

Semi 3D assays (Transwell invasion assay and Platypus invasion assay): The invasive capabilities of the cells can be assessed by adding a layer of ECM matrix to the porous membrane or directly over the attached cells to the transwell invasion assay [478] and platypus invasion assay (as described before) respectively. This modified transwell and platypus assays are not true 3D assay as the cells still have contact with the plastic and are not completely in the 3D environment. Semi 3D assays are quantified by calculating the so-called 'invasive index'. The ratio of invaded cells (cells which passed through the ECM matrix) against the migrated cells (assay without the ECM matrix) determines the relative contribution of invasion to the overall motility [479]. Since semi 3D assays are based on the principles of migration assays, the advantages and disadvantages related to the assay remain the same as the transwell migration assay and platypus migration assay.

Gelatin degradation assay: This assay provides high-resolution data on the invasive behavior of the cells at the sub-cellular level rather than the whole cells, which led to the discovery of cellular protrusions called invadopodia and podosomes [480]. The cells are seeded on top of a thin layer of fluorescently labeled ECM matrix. The invading cells degrade the matrix and create areas that lack fluorescence. **Quantification:** The fluorescence free areas are imaged and quantified by light microscopy. **Advantages:** High-resolution, sub-cellular information about the invading structure are obtained. Red or green fluorescently labeled gelatin as ECM matrix is commercially available. **Disadvantages:** The invasive properties of a whole cells cannot be followed. Furthermore, the cells are attached to a very thin layer of ECM and therefore may adapt a 2D cell shape instead of a real 3D structure [458].

Vertical gel invasion assays: In this assay, thick collagen plugs are prepared and cells are seeded on top of the gel. Radioactive labeling and scintillation counting are employed to monitor the vertical 3D migration of the cells [481]. This assays is often used to skin cancer cell invasion in organotypic skin model where the collagen layer is replaced with primary myoma tissue, which recapitulates the *in vivo* situation more closely [482]. **Quantification:** The collagen plugs with the invaded cells are formalin fixed and paraffin embedded (FFPE) which are then sectioned and stained immunohistochemically. **Advantages:** Mimics the *in vivo* situation for invasion. Cell-ECM interactions and cell-cell interactions can be monitored and studied. **Disadvantages:** This assay is not commercially available.

3D single cell tracking: Similar to the 2D cell tracking describe above, single cell suspensions can be embedded in ECM matrices and tracked in real-time. Confocal or multiphoton microscopy is used to track either labeled or unlabeled cells in 3D. The invasion of the directed by the stiffness of the 3D matrix, cell adhesion properties and proteolysis of the matrix is essentially demonstrated in the 3D cell tracking and not in the 2D cell tracking. Furthermore, the mode of cell motility and variations in the cellular protrusions can be investigated [483].

Spheroid invasion assay: Spheroid invasion assays are the most versatile and powerful among the 3D assays and there are various ways by which this could be performed. The prerequisite for this assay is the formation of multicellular aggregates of cells called the spheroids (Figure 10) [484]. In the *in vivo* scenario, the cancer cells invade the surrounding tissue from a cancer cell cluster. These spheroids exactly mimic the tumour clusters both in structure and ECM composition. The different variations of spheroid invasion assay are described below:

- **Spheroid gel invasion assay:** This method involves embedding the multicellular spheroids in 3D ECM such as collagen or matrigel. If the multicellular spheroids are composed of non-invasive cancer cells, they stay as compact spheroids within the matrix, but if the spheroids are composed of invasive cell lines, they invade into the surrounding matrix and display astral growth from periphery of the spheroid. In a very similar assay, cells coated on micro-carrier beads can be embedded in ECM matrix [485]. **Quantification:** Invasion is assessed as time-lapse or endpoints by live imaging or photomicrographs respectively. The gels with the invaded cells can be fixed and processed for immunofluorescence staining and imaged using confocal microscopy. Alternatively, the cells can be dissociated from the gel by enzymatic lysis of the matrix and the isolated cells can be analyzed with flow cytometry. **Advantages:** Closely mimics the invasion of cancer cells *in vivo* as the invasion occurs with well-established cell-cell interactions as opposed to single cells in other 3D assays. The assay can be adapted to 96 well and 384 well high throughput formats, which is of particular use in anti-metastatic / migration drug screening. **Disadvantages:** In cells coated on micro-carrier beads, it may be difficult to distinguish the real invasion from cell movement on the surface of the micro-carrier beads. Some spheroids may occasionally be at the bottom surface of the tissue

culture dish and the cells migrate away on the plastic giving false impression of rapid invasion of a particular spheroid.

- **Spheroid co-culture invasion assay:** This variation of the spheroid invasion assay is a model to study the invasive behavior of a certain cell type (lets say 'A', which most cases is a malignant cell) into a tissue like structure made of a different non-malignant cell type (cell type B). The assay is performed by co-culture of spheroids of cell type B with single cell suspensions of cell type A. The cell type A attached to the surface of the spheroid begins to invade inwards into the spheroid, which is made of cell type B [486]. **Quantification:** 3D invasion of fluorescently labeled cells can be analyzed by fluorescence microscopy. Alternatively, the cells can be dissociated and analyzed by flow cytometry or fixed, paraffin embedded and sectioned for immunofluorescence or immunohistochemical analysis. **Advantages:** It mimics the tightly arranged multicellular 3D structures and cell-cell interactions, as it is present *in vivo*. **Disadvantages:** Both cell types used in this model must be capable of forming compact spheroids. The quantification is not straightforward and it usually is labor intensive.
- **Spheroid confrontation assay:** This model involves two spheroids derived from different cell types cultured side by side and are eventually fused together to assess the interaction and invasive properties of the cell types. One of the cell types used is generally non-malignant (e.g. normal fibroblasts) and the other type is an invasive cancer cell line. Following embedment in the ECM matrix, the cells either infiltrate the opposing cell types or invade the matrix [487]. As this assay resembles the spheroid co-culture assay, the quantification, advantages and disadvantages remain the same.

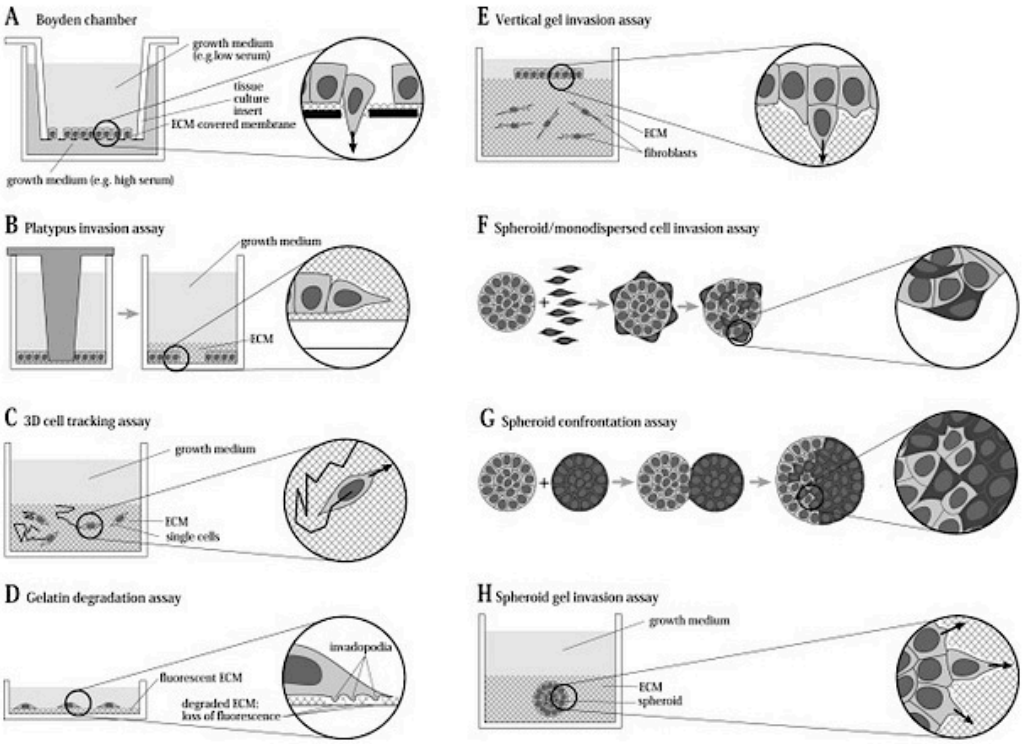


Figure 10: Schemes of commonly used 3D invasion assays. An overview of the technical setup is schematically drawn for each assay and a close up view of the invading cells is represented inside the circles. Arrows indicate the direction of cell movement. Crossed grey areas symbolize ECM. (Adapted from [458]

Applications of 3D invasion assays:

- *Cell Function:* The growth kinetics (size versus time), composition and other cell specific aspects like proliferation, differentiation, apoptosis, protein and gene expression of the invading cells can be assessed. Studies comparing gene expression of invading cells in spheroids and 2D cultures have reported significant differences in the genes related to cell survival, proliferation, differentiation and response to therapy, thus showing that spheroids more closely resemble *in vivo* tumours [488].
- *Drug screening:* Cancer spheroids are widely used to assess tumour responses and sensitivities pertinent to chemotherapeutics, combination therapies, targeted therapies and drug delivery vehicles. Spheroid cultures can easily be scaled-up to high-throughput platforms, which are used for negative selection of drug candidates to reduce animal testing and positive selection for new drug development [489].
- *Angiogenesis:* The potential for tumour vascularization is often assessed from the migration potential of endothelial cells into tumour spheroids or the formation of vascular networks within tumour spheroids [490].

1.6.2 Use of Patient derived xenografts in 2D and 3D migration / invasion assays

Continuous efforts have been made to better mimic the *in vivo* complex scenario in order to make *in vitro* models more reliable. However the 2D and 3D cell migration / invasion assays still harbor its limitations and further advancements are required to enhance the accurate usability of these models [491]. One of the key causes for low success rate of oncology drugs is due to the use of established cancer cell lines, which are in culture for decades in *in vitro* models. Historically, a small subset of cancer cells are propagated as continuous cell lines and used to identify targets or develop drugs in *in vitro* and *in vivo* models. However, due to lack of heterogeneity in the cell lines, they do not recapitulate and exemplify the preclinical predictions [492]. Patient derived xengrafts (PDX) may overcome some of these concerns and better mimic the *in vivo* situation in the 2D and 3D pre-clinical models (Figure 11) [493].

PDX are established by transferring fresh tumour tissue from patients into immuno-compromised mice. After a period of lag phase, the xenografts propagate and enter the log phase of growth, which are suitable for harvesting and be used in *in vitro* models or re-implanted in successive generations of mice. PDX models maintain the intratumour heterogeneity as seen in the primary tumours of the patients [494, 495]. Cell lines continuously propagated in the lab undergo extensive evolutionary selection through years and rarely recapitulate the histological parameters of the primary tumour, but PDX models are shown to sustain the histological features over time. PDX models maintain a complete spectrum of molecular subtypes of cancers, unlike the cancer cell lines that skew the subtypes to increase the affinity of growth in *in vitro* [496].

Although, PDX models are definitely a step forward in creating more clinically relevant pre-clinical models, there are some uncertainties associated with it, which are listed below and summarized in the table:

- PDX models maintain the component of human stroma in early passages, but in later passages the human stroma is replaced by the murine stroma. Human derived xengrafts are supported by the murine stroma, but there may be changes in the gene expression of the PDX from early to late passages and the effect of these changes needs to be addressed [492].
- Many PDX models are generated from the primary tumours and thereby fail to emulate the chemotherapy-refractory and metastatic patient population in whom most novel therapeutics undergoes initial trials [496].
- An increasing number of cancer based clinical trials incorporate immune-based therapies, which questions the use of immunosuppressed mice for establishing PDX models [497]. The influence of immune cells on the drugs tested cannot be determined.

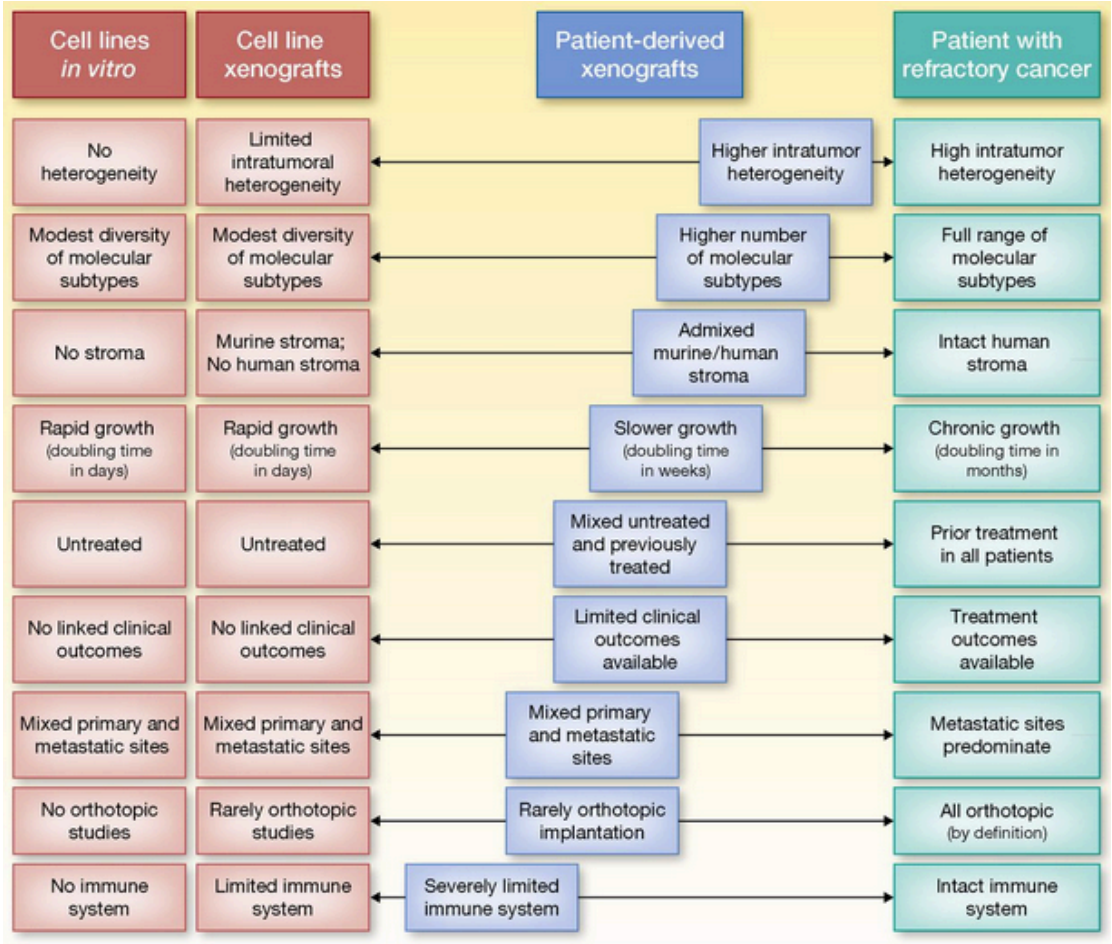


Figure 11: Advantages of PDX models as compared to established cell lines *in vitro* and cell lines derived from xenografts. (Adapted from [492])

The advents of tumour bio banks around the world and efforts to pair the clinical outcomes from patients and response of the corresponding PDX have shown early success [498, 499]. Validation of PDX models will enable successful selection of critical biological phenomenon amenable to pharmaceutical intervention thereby reducing early drug development failures.

1.6.3 Ex vivo co-culture cell motility systems

From monolayers to 3D cultures, a wealth of information was provided about the cells' migratory behavior in a close to physiologically relevant context. However, tissue-specific architecture, mechanical and biochemical cues and cell-cell communication are lost under such simplified and highly biased conditions. Thus organotypic slice cultures have been established to study the cellular functions in the cells' own architectural niche and to achieve more *in vivo* like situations. While organotypic slice cultures were established for different tissues, the first documented report on 'organotypic' cultures was on differentiation of chick embryo eye followed by reports on lung, heart and intestine [500]. Typically, the slice cultures involve sectioning the tissues (approximately 400-500µM thick) of interest from 2-23 days old mice and the slices are maintained in culture *in vitro* [501]. Though there are various methods of organotypic slice culture, the most commonly used method is the Stoppini's method, which involves the culture of the slices on a semi porous membrane [502]. Organotypic cultures of the brain are one of most extensively used slice cultures, with a view to study the cell behavior in the native brain architecture. In general, slices cultured can be studied immediately after dissection or after being cultured for longer times. Studies immediately after the dissection may display a near *in vivo* situation [501].

In oncology, tumour cells are seeded on top of the cultured organ slices to establish an *ex-vivo* co-culture system for assessing the tumour cell behavior (proliferation, growth and migration) in a more complex *in vivo*-like environment. [503] Brain slices are of particular interest because they not only provide the brain's native architecture for the tumour cells but also the vascular structure of the brain. Brain slices contain a dense network of brain capillaries, which can be detected by immunohistochemical positivity for laminin. Though, the slices are devoid of any blood flow and the capillaries no longer function, it is probable that they maintain the factors and cells in the neurovascular unit. In addition, the brain slice cultures can also aid in studying the BBB dynamics and its influence on drug availability in brain tumours. However, there is no functional *ex-vivo* BBB model to assess this phenomenon [501].

Organotypic brain slices can be analyzed using all common biochemical methods such as ELISA, RT-PCR (after dissociation of cells) or by immunofluorescence after fixing and staining the slices. Organotypic brain slices are used to conduct neuro-protective and neuro-toxic assays (drug screening), where the effects of growth factors and drugs are assessed respectively. Recently the effects of pro-angiogenic and pro-migratory factors like VEGF, bFGF and its influence on tumour cell invasion in slice cultures are being evaluated [504]. Taken together, organotypic slice cultures need to be optimized to study cell invasion and migration and means to quantify migration / invasion in this complex environment has to be developed. Thus, slice culture model is the closest to an *in vivo* situation and significantly reduce the number of animals required for *in vivo* experiments.

1.6.4 In vivo models to study cell motility / invasion

2D, 3D and *ex vivo* models strive hard to imitate *in vivo* scenarios, the fact that 'there is no place like *in vivo'* is undeniable. However, The initial infiltration and 'getaway' mechanisms of metastatic cells have been less studied *in vivo*. This is due to the relative inaccessibility of this process in live animals.

Intravital-imaging studies are the most advanced models used for characterizing primary tumour properties, growth rates and mechanisms of metastasis to distant organs directly in live animals [505]. Intravital imaging studies combines advanced optical platforms like laser-scanning microscopy with long wavelength and multiphoton fluorescence excitation to capture high-resolution, three-dimensional images of the living tissue, which are tagged prior with highly specific fluorophores [506, 507]. Apart from the fluorophores, current intravital imaging also includes the use of quantum dots for labeling live tissues. The new generations of quantum dots have far-reaching potential into the tissue of interest thus enabling the study of intracellular processes at a sub-cellular single molecule level resolution. Further advancement in this technique involves the use of animal models of cancer (orthotopic or genetic models) that stably express a fluorescent protein such as Green fluorescent protein (GFP) [508]. These advances aid in new insights about the mechanism of cell migration during intravasation and the influence of microenvironment on the key steps of metastasis. Intravital imaging also facilitates direct visualization of entry of tumour cells into circulation, which has always been assayed via indirect methods using tumour cell markers in the blood.

Intravital imaging has been indispensible to identify the several differences between the behaviors of carcinoma cell migration *in vivo* as compared to *in vitro* models. The differences are observed in mode of migration, velocity and directionality. In mammary tumours, cells migrate as solitary amoeboid cells as opposed to collective migration observed in *in vitro* models [509]. Similarly, adenocarcinoma cells migrate at speeds tenfold higher than those observed *in vitro*. In addition, metastatic cells show a more directional locomotion *in vivo* as compared to the non-polarized random movements in 2D environment. These differences will in-part help to identify the tumours for which 'anti-metastatic' therapy might be suitable. For inoperable and highly metastatic tumours, treatments that would obstruct intravasation could prolong the patients' life. These treatments could be helpful in restricting further spread of the disease independently of their ability to reduce the size of the primary tumour [510].

Intravital imaging is the most realistic approach to obtain an overall idea on the *in vivo* cell migration. However, there are also *in vivo* models that enable the defragmentation of invasive cells from the primary tumour. This *in vivo* invasion assay takes advantage of the chemotactic properties of cancer cells to determine invasive properties of cancer cells *in vivo*. This assay involves microneedles being filled with ECM proteins or matrigel with or without a chemoattractant and then introduced into the primary tumour (orthotopic or genetic model) of a rat or mouse. The animal is kept under anesthesia over duration of 4 hours following which the invasive cells enter the needle. The invasive cells are isolated from

the microneedles and can be analyzed in terms of number, cell type, gene expression profile and response to different stimuli [511]. Thus, this assay provides a snapshot of genomic / proteomic profile of those cancer cells that have the propensity to migrate and invade. This assay can be made clinically relevant by including pharmacological inhibitors in the needle thereby novel targets and signaling pathways contributing to *in vivo* invasion are identified.

1.7 MAP4K4

Background – MAPK:

Mitogen-activated protein kinases (MAPK) are constituents of a highly conserved cascade of serine / threonine kinases that have a distinctive Thr-x-Tyr (here 'x' can be any other amino acid) motif within its kinase activation domain and are key players in many signal transduction pathways [512]. MAPKs have been to shown to be involved in inflammation, stress response, migration and oncogenesis. In general, kinase-signaling pathways promote many of the hallmark phenotypes of tumour biology such as proliferation, survival, motility, metabolism, angiogenesis, invasion and evasion of anti-tumour response [513]. This indicates that kinases can be excellent targets for novel cancer drug development. Kinases are very straightforward to target as all kinases have a conserved activation loop. The activation loop is central to the regulation of kinases activity and is marked by well conserved DFG and APE motifs. The activation loop can assume a large number of conformations ranging from 'active' phosphorylated form to 'inactive' conformation [514]. Small molecule inhibitors, which are capable of binding to the activation domain or ATP binding domain, can be designed to sustain the kinase in the inactive conformation thus disrupting the signaling cascade [515].

The MAPK family is classified into three broad groups based on the type of activation loop namely, extracellular signal-regulated protein kinase (ERK/MAPK), which has a Thr-Glu-Tyr motif; p38 with the Thr-Ala-Tyr motif and JUN N-terminus kinase (JNK), which harbors Thr-Pro-Tyr motif [513]. The MAPK cascade with its versatile role in translation of distinctive extracellular signals to diverse physiological responses and its huge potential as a targeted cancer therapy has led to the investigation of new upstream kinases that interconnect and modulate the downstream effector MAPK kinases. This led to the identification and characterization of new class of MAP4Ks and MAP4K4 in particular.

MAP4K4 – Mitogen Activated Kinase kinase kinase kinase 4 – activity and regulation:

MAP4K4 (also known as hepatocyte progenitor kinase-like / germinal center kinase like kinase (HGK) or Nck interacting kinase (NIK)) with ≈ 1200 amino acids and a molecular mass ≈ 140KDa is a serine / threonine kinase. It belongs to the mammalian family of Ste20 protein kinases because of their shared homology to the *Sacchromyces cerevisiae* kinase Ste20 [516]. Based on the location of the catalytic domain, the Ste20 family can be broadly divided into p21-activated kinases (PAKs, C-terminus) and germinal center-like kinases (GCKs, N-terminus). MAP4K4 belong to one of the four members of the GCK-IV subfamily [517]. MAP4K4 is expressed in all tissue types but are proportionally higher in brain and testis

[518]. There are five alternatively spliced transcript variant isoforms of MAP4K4 with identical kinase domain at the N-terminus and differences in the intermediate domains. The citron homology domain (CNH) at the C-terminus of MAP4K4 determines its association with other factors and thus considered as regulatory domain of MAP4K4 [519]. MAP4K4 activates JNK by its kinase activity and via its C-terminal regulatory domain that mediates the interaction of MAP4K with Mitogen activated protein kinase kinase kinase 1 (MEKK1). MAP4K4 can modulate other proteins independent of its kinase activity, thus excreting scaffolding functions. The interaction of MAP4K4 with Signal transducer and activator of transcription 3 (STAT3) and proline rich tyrosine kinase 2 (PYK2) does not require the catalytic kinase activity of MAP4K4. The putative interactors of MAP4K4 are identified via co-immunoprecipitation and mass spectrometry, which provides little information about the kinase activity [520]. However, like other kinases MAP4K can be positively or negatively regulated by upstream kinases and these kinases remain largely unidentified. It is highly probable that the kinase activity of MAP4K4 could be dependent on cell type, external stimuli and cell state.

1.7.1 MAP4K4 in cancer

Evidences form gene expression profiles and correlation studies with MAP4K4 suggested the role of MAP4K4 in cancer. MAP4K4 is over expressed in 40 out of a 60 NCI tumour cell lines panel and is shown to modulate cell growth, invasion and adhesion via STAT3 [518]. MAP4K4 expression is a negative predictor of overall survival, early recurrence rate and lymph node metastasis in hepatocellular carcinoma [521], lung carcinoma [522], prostate cancer [523] and colorectal cancer tissues and cell lines [524]. Downregulation of MAPK4K4 with siRNA or shRNA decreased cell proliferation, colony formation, migration, invasion, xenograft tumour growth and anchorage independent growth, while G0/G1 arrest, apoptosis, cell adhesion and chemosensitivity was increased in a wide range of cancer such as colorectal cancer, gastric cancer, pancreatic cancer, ovarian carcinoma, giloblastoma and kaposi's sarcoma [515].

Despite the aforementioned role of MAP4K4 in modulating cancer cell behavior, little is known about the downstream effectors of MAP4K4 that mediate its biological functions. Indications from knockdown studies showed that the gene expressions of plethora of effectors are affected. These factors are kinases like MAPK/JNK [476]; transcription factors such as NF-κB, STAT3 and HES1 [525], transmembrane receptors like Notch2 and Notch, matrix metalloproteinases such as MMP-2, MMP-9, MMP-7 and MMP-13 [526], inhibitors of apoptosis, negative regulator of p53 (MDM2) [527], inflammatory-related factor cyclooxygenase-2 and toll-like receptors [528]. However, all of the above effectors are not modulated by MAP4K4 through canonical MAPK pathways as expected, suggesting that MAP4K4 may contribute to cancer through MAPK-independent mechanism. Furthermore, the mechanism by which MAP4K4 modulates its downstream effectors is not well understood. Indeed, MAP4K4 could exert its kinase activity by direct phosphorylation of its substrates, but none of these effectors are directly phosphorylated by MAP4K4 in cancer [515].

In addition to the MAPK dependent and independent pathways, MAP4K4 is also involved in non-MAPK pathways like the insulin signaling pathway [529], the hippo pathway (LATS1/2 and YAP/TAZ) [530, 531] and the mTOR pathway [532]. Although, the role of MAP4K4 in cancer via these pathways remains to be elucidated, it is likely that MAP4K4 can contribute to cancer via modulating these pathways. To date, TNF-α [533] and EGF are the only documented upstream activators of MAP4K4. ERK-MAP4K4 cascade phosphorylated ERM proteins and promotes cell migration [534].

1.7.2 MAP4K4 in cancer cell migration

Studies on developmental processes dominated by cell migration like dorsal closure in *Drosophila* and migration of mesodermal cells during gastrulation in mice, demonstrated that MAP4K4 is required for cell migration [535]. Downregulation or knockdown of MAP4K4 impeded the cancer cells' ability to migrate, implying its role in cancer cell migration [536]. However, the pathway for MAP4K4 induced cell migration is not clear as there are controversial studies showing the participation of both JNK and p38 signaling cascades.

1.7.3 Targeting MAP4K4

In line with other cancer-associated kinases, MAP4K4 with its diverse role in cancer is a lucrative therapy target. GNE-495, GNE-220, PF-6260933 and 4-hydroxy-2-pyridone are the MAP4K4 specific small-molecule inhibitors currently available [537]. Some of these inhibitors inhibit angiogenesis in mice. Other potential anti-tumour properties of these inhibitors are elusive, but if proved, MAP4K4 inhibition may be a novel targeted therapy in cancer [515].

1.8 Fibroblast growth factor receptor signaling

The signaling component of mammalian Fibroblast Growth Factor (FGF) comprises of 18 secreted proteins that interact with 4 tyrosine kinase FGF receptors (FGFRs), which controls a wide range of biological functions including regulating cellular proliferation, survival, migration and differentiation [538].

1.8.1 Types of fibroblast growth factors

The FGF superfamily consists of 23 members and the function of FGFs is not restricted to promotion of fibroblast growth. In that sense, 'fibroblast growth factor' is a limited description for this family of cytokines. All FGFs have a conserved core region made up of 120 amino acids (aa) that contains six identical, interspersed amino acids [539]. The FGF superfamily can be broadly divided into secreted FGFs (also called as canonical FGFs or paracrine FGFs) that signal to receptor tyrosine kinases and intracellular FGFs (iFGFs) that serve as cofactors for voltage gated sodium channels. Secreted FGFs are expressed in nearly all tissues and function as autocrine or paracrine factors thus controlling essential roles in the earliest stages of embryonic development, during organogenesis, maintenance of homeostasis, wound repair, regeneration and metabolism in adults [540]. The intracellular FGFs function as endocrine factors where they regulate phosphate, bile acid and

carbohydrate and lipid metabolism and like secreted FGFs control cell proliferation, differentiation and survival [541].

FGF1 subfamily: FGF1 subfamily consists of the most abundant FGFs namely FGF1 and FGF2 (also called bFGF). FGF1 subfamily functions through the direct translocation across the cell membrane mediated by synaptotagmin-1 and the calcium binding protein S100A13. This direct translocation is indispensible for FGF1 and FGF2 activation as they lack the secretory signal peptides [542]. FGF1 and FGF2 are translocated to the nucleus mostly via their binding to the cell surface tyrosine kinase FGFRs with heparin / HS and HSP90. FGF1 subfamily contributes to the regulation of cell cycle, cell differentiation, survival and apoptosis [543-545]. The structure and functions of the members of FGF1 subfamily are explained below:

- *FGF1:* Human FGF-1 (also known as FGF acidic, FGFa, ECGF and HBGF-1) is a 17-18 KDa non-glycosylated polypeptide that is expressed in nearly all the cells including intestinal enterochromaffin cells, renal proximal tubule cells, smooth muscle cells, neurons, hepatocytes, skeletal muscle cells, endothelial cells, macrophages, keratinocytes and fibroblasts from all three germ layers [546]. Consistent with the features of FGF1 subfamily, FGF1 has no signal peptide and is released as disulfide-linked dimer. FGF1 is the only FGF, which is known to interact with all the 4 FGFRs. In addition to the activation of FGFRs, FGF1 can also function intracellularly via its nuclear localization sequence (NLS) [547]. The path FGF1 takes to the nucleus is not known but once localized in the nucleus it contributes to DNA synthesis [548].
- *FGF2:* FGF2 (also known as FGF basic, HBGF-2 and EDGF) is also a non-glycosylated polypeptide secreted as a monomer [549]. Secreted as an 18 KDa protein (155 aa in length), FGF2 is sequestered after secretion on cell surface heparin sulphate (HS) or matrix glycosaminoglycans [550]. Despite being secreted as a monomer, cell surface HS dimerizes FGF2 in a non-covalent side-to-side configuration that subsequently dimerizes and activates FGF receptors. Similar to FGF1, FGF2 in association with HS or FGF receptor is internalized and can either be degraded into 4-10 KDa bioactive fragments or translocated to the nucleus. Although FGF2 has a NLS at the N-terminus, it enters the nucleus predominantly via the methylation of arginines 24-28 aa upstream of the first methionine. Nuclear translocated FGF2 induced effects such as casein kinase II activation, which in turn regulates cell-cycle progression and proliferation [551]. A wide range of cells is known to express FGF2. They are visceral and vascular smooth muscle cells, cardiac muscle cells, lining epithelium of colon and bronchus, neurons, cerebellar Purkinjie cells, megakaryocytes, platelets, endothelial cells, mast cells, glomerular parietal epithelia cells, podocytes, astrocytes, CD4 T cells, CD8 T cells, fibroblasts (in ECM) and numerous embryonic mesodermal and neuroectodermal tissues [549].

FGF4 Subfamily: Based on the phylogenetic analysis FGF4, FGF5 and FGF6 are grouped under the FGF4 subfamily [552]. These FGFs possess a cleavable N-terminal signal peptide and are secreted to mediate the biological responses via binding and activating the IIIc splice variant of FGFRs1-3 and FGFR4 [553].

FGF7 Subfamily: This subfamily of FGFs preferentially activates the IIIb splice variant of FGFR2 and IIIb splice variant of FGFR1 and is comprised of FGF3, FGF7, FGF10 and FGF22 [552, 553].

FGF8 Subfamily: This subfamily includes FGF8, FGF17 and FGF18. All FGFs of this family has a N-terminally cleaved signal peptide and activate IIIc splice variants of FGFR1-3 and FGFR4 [552, 553].

FGF9 Subfamily: In contrast to the other FGF families, FGF9 subfamily does not have a classical N-terminal signal peptide. They are characterized by the presence of an internal hydrophobic sequence, which functions as a non-cleaved signal for transportation to the endoplasmic reticulum and secretion for cells [554]. FGF9, FGF16 and FGF20 are classified under this subfamily and all the members of this family exclusively activates IIIb splice variant of FGFR3, splice IIIc variant of FGFR1-2 and FGFR4 [553].

FGF15/19 Subfamily (Endocrine FGFs): This subfamily of FGFs function as endocrine factors and are comprised of FGF15/19, FGF21 and FGF23. In contrast to the secreted FGFs, endocrine FGFs bind to heparin with low affinity facilitating its release from ECM to function as endocrine factors. Although, these FGF function as endocrine factors, they still signal via FGFRs by utilizing members of the Klotho family as cofactors for receptor binding and activation [555]. Klotho family (αKlotho, βKlotho and Klotho-LPH related protein (KLPH)) are structurally related single-pass transmembrane proteins of ≈1000 aa with a short cytoplasmic domain. FGF15/19 and FGF21 specifically require βKlotho and can activate FGFR Ic, FGFR 2c, FGFR3c and FGFR4, while FGF21 activates only FGFR1c and FGFR3c [556]. FGF21 is implicated in hepatocyte and adipocyte metabolism while FGF19 specifically activates FGFR4 and is involved in regulation of bile acid synthesis and is associated in the progression of hepatocellular carcinoma [557].

FGF11 Subfamily (Intracelluar FGFs (iFGF)): As the name suggests, this subfamily of FGFs are not secreted and does not interact with FGFRs. This subfamily comprises of FGF11, FGF12, FGF13 and FGF14 [558].

1.8.2 Types and specificity of fibroblast growth factor receptors

Four distinct fibroblast growth factor receptors (FGFRs) are identified to date and classified as FGFR1-4. FGFRs belong to the receptor tyrosine kinase (RTK) superfamily and due to high homology between all FGFRs; they are classified as a new subfamily among the superfamily. All FGFRs share a common unique structural backbone with an extracellular, ligand-binding domain, a single transmembrane domain and cytosolic, tyrosine kinase domain (Figure 12) [559, 560].

- **Extracellular ligand-binding domain:** This domain is composed of three immunoglobulin (Ig) like domain and an acidic box of eight aa (mainly Asp or Glu) sandwiched between the first and the second Ig like domain.

- **Transmembrane domain:** This domain is an 80-residue long transmembrane region attached to the extracellular domain. The transmembrane domain of FGFRs is relatively long as compared to approximately 60 residues in other classes of RTKs.
- **Tyrosine kinase domain:** This is the cytoplasmic domain responsible for the kinase activity of the receptor. The COOH-terminal tail contains the tyrosines, which are phosphorylated upon ligand binding. FGFRs have a short kinase insert composed of only 13 aa as compared to approximately 78 aa long insert in PDGFR.

The binding of FGFRs with specific FGFs is a complex phenomenon due to tissue-specific alternative splicing in FGFR. Alternative splicing allows for the generation of two distinct receptors from a single gene representing a novel genetic mechanism to generate receptor diversity [561].

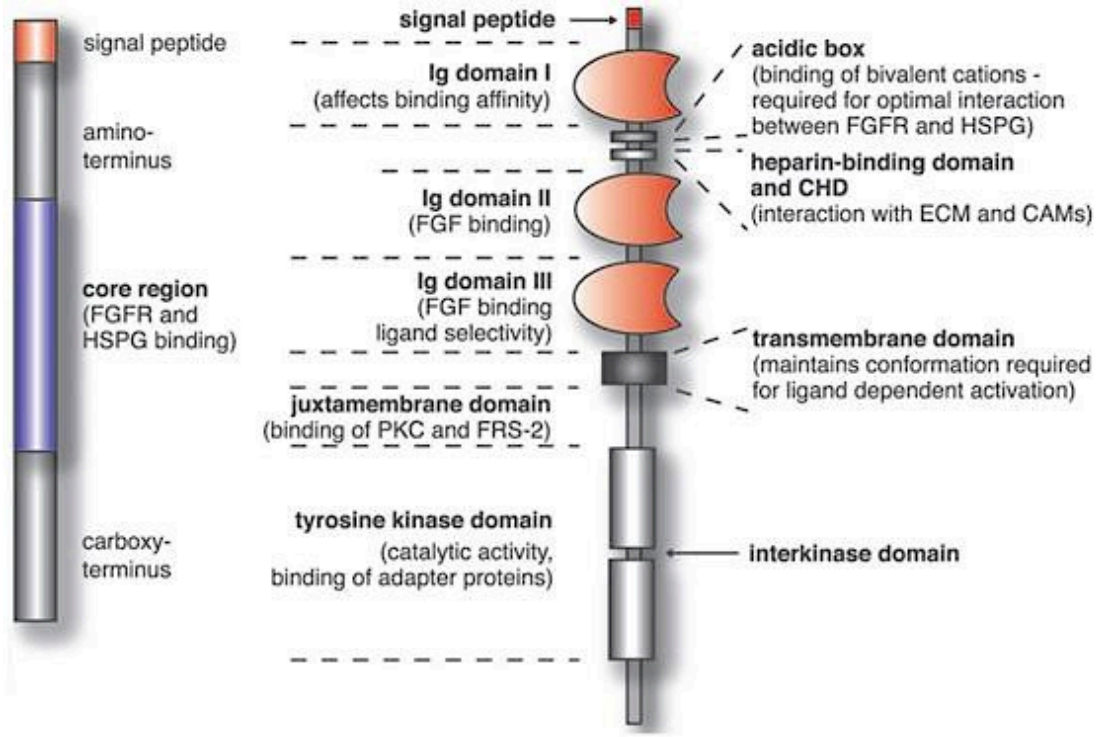


Figure 12: Structure of FGFR: Schematic representation of general structure of FGFR with distinct domain regions. (Adapted from [562])

Alternative splicing of FGFRs:

The FGFR1 contains 19 exons that spans approximately 20Kb. The exons and the corresponding domains they code are closely related but each domain is coded by a separate exon. The extracellular domain (Ig-like domain) is coded by 9 exons. Within the three Ig-like domains, domain 1 is coded by exon 3 and domains 2 and 3 are each encoded by two other exons. The N-terminal end of domain 3 is coded by non-variable, 'constant' exon IIIa whereas the C-terminal part of domain 3 is duplicated and coded by the 'variable' exons (exon IIIb and exon IIIc). Therefore, alternative splicing can generate Ig domain 3 either from exon IIIa and exon IIIb or exon IIIa and exon IIIc. A similar exon rearrangement and splicing is also observed in FGFR2 and FGFR3. In the case of FGFR2, the splice variant from exon IIIa and exon IIIc encode the classical FGFR2 and the variant from exon IIIa and

exon IIIb encode KGFR. In addition to the choice between exon IIIb and exon IIIc, other splice variant of FGFR devoid of Ig domain 1 are also identified. These splice variants express Ig-like domains 2 and 3 and are membrane bound. The secreted FGFR binding domains can functionally inhibit FGFR signaling [563]. Ig-like domain of FGFR4 is not alternatively spliced [564]. Among the FGFRs, the alternative splicing of FGFR2 is functionally most important, as it is required in the early developmental stages. This genetic mechanism of alternative splicing of FGFRs well conserved in species ranging from sea urchins to mammals [565].

Ligand binding affinity and specificity of FGFRs:

The binding specificity of the 18 secreted FGFs with the alternatively spliced variants of FGFRs is measured using mitogenic assays performed in BaF3 cell lines. BaF3 cells are a good model systems for FGFR mitogenic assays as they have no or negligible FGFR expression. FGFR1 and FGFR2 responded strongly to mitogens, whereas FGFR3 and FGFR4 render weak responses [553]. This suggests that the strength and downstream pathways activated by the different FGFRs will be unique for each receptor and splice variant.

Tissue-specific regulation of FGFRs controls the expression of alternative splice variants of FGFR1 and FGFR2. Mesenchymal tissues express FGFR1 III and FGFR2 IIIc and are surprisingly activated by FGFs like FGF4 and FGF8, which are expressed in the epithelial cells. Similarly, epithelial cells express the splice IIIb variant of FGFR1 and FGFR2 and are activated by the mesenchymal specific FGFs like FGF7. This reciprocal expression and interaction of FGFRs and FGFs are indispensible for organogenesis, particularly those that undergo branching morphogenesis such as the lung or salivary gland and structures like the limb bud and skin [553, 566]. FGF1 is the only ligand that could activate all FGFR splice variants. A detailed list of all the FGFs and its specific binding affinities to the various splice variants of FGFRs are summarized in the Figure 13:

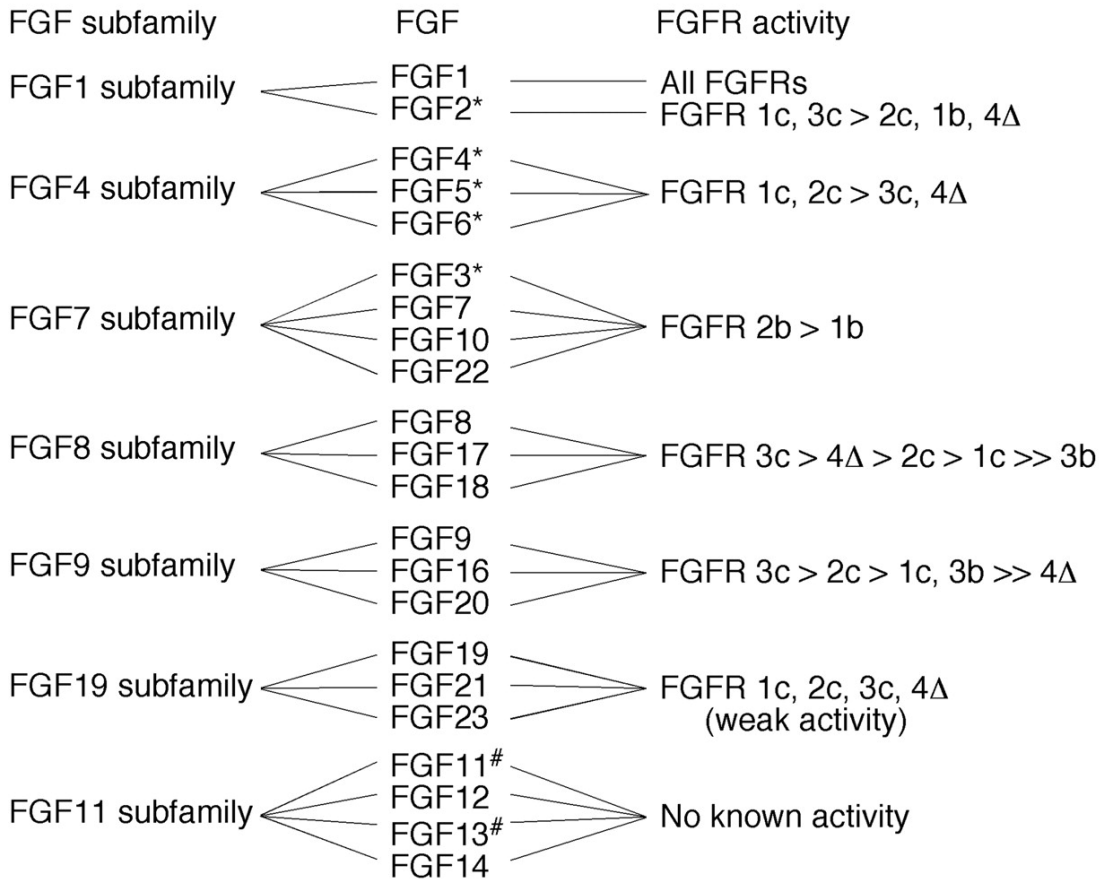


Figure 13: Specificity of FGFRs: Diagram showing the relative affinity towards FGFs grouped by FGF subfamilies. * - data from ([566]). # - data not tested by ([566]). (Adapted from [553])

1.8.3 Regulation of the FGFR pathway

Extracellular positive regulators of FGF signaling pathway:

Heparan sulphate proteoglycans: HS is a potent cofactor for the canonical FGF signaling. HS is composed of a long linear carbohydrate chain of repeated, sulfated disaccharides and glucuronic acid linked to N-actylglucosamine. Specific core proteins like syndecan, perlecan, glypican and argin are covalently linked to HS chains. HS is capable of interacting independently with both FGFs and FGFRs. HS specifically binds to the cleft between the N-terminal regions of Ig-like domain 2 of FGFs thereby cooperatively increasing the affinity of 1:1 FGF-FGFR. The resultant 1:1:1 FGF-HS-FGFR complex undergoes a conformational change that stabilize a symmetric 2:2:2 dimer. FGFR dimerization then activates the intracellular signaling pathways by juxtapositioning and activating the tyrosine kinase domains [567, 568]. In contrast to its aid in activating FGF signaling, HS is also responsible for sequestering FGFs. HS present in the ECM sequesters FGFs and modulate their diffusion through tissues. These modulations are essential for organogenesis, which can be demonstrated by the differences in binding affinity of FGF7 and FGF10 during epithelial branching patterns [569].

The sulfation patterns and length of HS chains regulate FGF signaling [568]. In general, higher levels of sulfation of HS chains positively correlate with FGF pathway activation and formation of ternary complexes with FGFs and FGFRs. In addition, cleavage of HS core

protein also influences FGF signaling by releasing FGFs that were sequestered at the cell-surface [570].

Klotho family proteins: The identification of αKlotho-FGF23-FGFR signaling in the kidney was the first evidence of Klotho family proteins as cofactors of FGF signaling. It is well documented that Klotho proteins act as cofactors for the endocrine FGFs through the formation of FGF-FGFR-Klotho ternary complex. Nevertheless, Klotho proteins also directly compete with a receptor-docking site for FGF8 thereby actively suppressing canonical FGFs while activating endocrine FGFs [571].

FGF binding proteins: FGF binding protein 1 (FGF binding protein 1) is a 234 aa polypeptide that binds to heparin, FGF1 and FGF2. FGFBP1 when bound to FGF, mobilize FGF from HS binding sites in the ECM and presents FGF to FGFR. FGFBP1 binds to and activates FGF7, FGF10 and FGF22 and enhances wound healing [572]. Naturally, as it is implicated in wound healing, it is expressed in several human tumours such as breast and colon cancer. FGFBP1 is pro-angiogenic and promotes tumour invasion by limiting tumour growth [573].

Negative regulators of FGF signaling:

Sprouty (SPRY): The sprouty family of proteins consisting of SPRY1-SPRY4 is a board, intracellular negative regulator of RTKs including FGFR, VEGFR, PDGFR and nerve growth factor receptor (NGFR) [574]. SPRY proteins share a conserved C-terminal cysteine-rich domain and an invariant tyrosine phosphorylation site at the N-terminus, necessary for their specific localization and protein-protein interactions [575]. In FGF signaling, SPRY inhibits the RAS-MAPK pathways and regulates the PLC-Y pathways. SPRY interacts with GRB2, thus blocking the association of GRB2 with FRS2 and FGF-induced signal transduction is repressed. SPRY2 regulates PLC-Y by directly binding to Raf via Raf-binding motif in their C-terminal domain and suppressing the phosphorylation of Raf on Ser338 by the Raf kinase PKCδ. Thus deregulation of SPRY often results in the constitutive activation of FGFR signaling leading to the formation of tumours [576].

Similar expression to FGF (SEF): SEF is an antagonist of RAS-MAPK pathway activation by FGF. This transmembrane protein inhibits the dissociation of MEK-ERK1/2 by binding to activated-MEK, thus preventing the nuclear translocation of ERK1/2. In addition, SEF may interact directly with FGFR through its extracellular domain to inhibit receptor phosphorylation [577-579].

Dual-specificity phosphatase 6 (Dusp6): Dusp6 encodes an ERK-specific MAPK phosphatase (MKP3) and negatively regulates FGFR signaling by directly dephosphorylating MAPK on phosphotyrosine and phosphothreonine residues [580].

CBL: CBL is an E3 ubiquitin ligase that tags FGFR and FRS2 for degradation by forming ternary complexes with phosphorylated FRS2α and GRB2. FGFR2 activation may also lead to increased CBL-PI3K interactions resulting in PI3K degradation and attenuated signaling [581].

FGFRL1 /FGFR5: FGF like receptor 1 (FGFRL1) is a protein structurally similar to FGFRs with three extracellular Ig-like domains, a single transmembrane domain and a short intracellular domain with no tyrosine kinase domain. The cytosolic domain of FGFRL1 contains a SH2 binding motif that interacts with tyrosine phosphatase SHP1. FGFRL1 binds to heparin and FGF2, 3,4,8,10 and 22 and evokes a cellular effect that ultimately leads to reduced growth and increased cell differentiation [582]. FGFRL1 is not a decoy receptor, but rather a non-tyrosine kinase-signaling molecule that could interact with other FGF family members and potentially inhibit FGF signaling [583].

ERK1/2: The ERK1/2 activated by FGFR-FRS2-RAS-MAPK cascade can exert a direct negative feedback inhibition of FGFRs. Tyrosine phosphorylation of FRS2 is a prerequisite for activating the RAS-MAPK cascade [584]. Activated ERK1/2 phosphorylates FRS2 on several serine / threonine residues, consequentially reducing the tyrosine phosphorylation of FRS2. Reduced phosphorylation of FRS2 leads to insufficient recruitment of GRB2 reduced MAPK activity and subsequent mitigation of FGF signal transduction [585]. There are three possible mechanisms by which serine / threonine phosphorylation of FRS2 attenuates FGF signaling:

1. Activated ERK binds FRS2 and interferes with its capability of to interact with FGFR. If FGFR and FRS2 interaction is disrupted, tyrosine phosphorylation of FRS2 is prevented, leading to reduced FGF signaling.
2. Ser/Thr phosphorylation of FRS2 may induce a structural change in FRS2, thus making FRS2 a poorer substrate for FGFR
3. Activated ERK and ser/thr phosphorylated FRS2 complexes may interfere with the interaction between the catalytic domain of FGFR and FRS2, whose interaction is a necessary step for the tyrosine phosphorylation of FRS2.

1.8.4 Effectors of FGFR signaling

Signaling pathways:

FGFs act as signaling ligand molecules that bind to and dimerize (activation – hetero or homodimers) FGFRs. Activated FGFRs triggers a number of signaling pathways leading to a specific cellular response via recruiting specific molecules that bind to phosphorylated tyrosine at the cytosolic part of the receptor. The phosphorylated tyrosine residues serve as docking sites for the recruitment of Src homology 2 (SH2) or phosphotyrosine binding (PTB) domains of adapter proteins or signaling enzymes. Downstream signaling complexes are formed and recruited to the dimerized activated receptors thus initiating a cascade of phosphorylation events [586]. FGF with its diverse functions is organogenesis and development, the plethora of its downstream effectors are vast. The best-understood and important FGF signaling pathways are explained below (Figure 14):

RAS/MAP kinase pathway: The MAPK pathway is the most common signaling pathway of FGF and regulates gene expression, mitosis, differentiation and survival. Activated MAPKs namely JNK, p38 and ERK are observed in response to FGF in all cell types. The first key event for MAPK activation by FGF signaling cascade is the tyrosine phosphorylation of the docking protein FRS2α [587]. This generates new binding sites for direct or indirect recruitment of

positive and negative regulators of FGF signaling. For the activation of FGF signaling, FRS2α recruits a complex consisting of the adapter proteins GRB2, SOS and SHP2 and the docking protein, GRB2 associated binding protein 1 (GAB1). The resultant FRS2 complex activates both the RAS/MAPK and PI3K/AKT pathways [588, 589]. The RAS/MAPK pathway is tightly regulated by both positive and negative mechanisms (see negative regulators of FGF) and subtle changes in the signals are important determinants of the biological response during development.

PI3 kinase / AKT pathway: Comparable to the RAS/MAP kinase pathway, the formation of the FRS2 signaling complex is the first event in the initiation of the PI3K/AKT pathway. GAB1 in the FRS2 signaling complex couples the activated FGFRs with p38 substrate of PI3 kinases. GAB1 contains a pleckstrin homology domain, a proline rich domain and SH2 binding domain with multiple tyrosine phosphorylation sites. PI3K with its p110 catalytic subunit form a complex with an adaptor protein (p85) that has two SH2 domains and binds to the phosphorylated tyrosine residues of GAB1. Phosphoinositide-dependent kinase and AKT are late downstream effectors of PI3k [590]. The PI3K/AKT pathway is implicated in cell survival and cell polarity control, which requires GAB1 for the stimulation of AKT by FGF [591].

PLCγ pathway: In contrast to the RAS/MAPK and PI3K/AKT pathways, activation of phospholipase C gamma (PLCγ) does not require the formation of the FRS2 signaling complex [592]. PLCY is activated by its associated with the phosphorylated Y766 residue of the activated FGFR. Activated PLCγ exerts hydrolysis of phosphatidylinositol, generating inositol triphosphate (IP3) and diacylglycerol (DAG). The second messenger DAG facilitates the release of calcium from the endoplasmic reticulum, which in turn leads to the increased levels of calcium in the cytosol. The increased levels of calcium and DAG activate the next effector in the cascade namely protein kinase C (PKC). This FRS2-independent pathway does not affect mitogenesis or cell differentiation but it is implicated in cell adhesion [593, 594].

STAT pathway: The activated FGFR also activates STAT1, STAT3 and STAT5 causing STAT pathway target gene expression. Constitutively activated mutant of FGFR3 activated STAT1 in chondrocytes [595, 596]. In cancer cells, gene amplifications and overexpression of FGFR3 causes STAT3-dependent gene expression. In brain microvascular endothelial cells, migration, invasion and tube formation are actuated by FGF activated STAT5 [597].

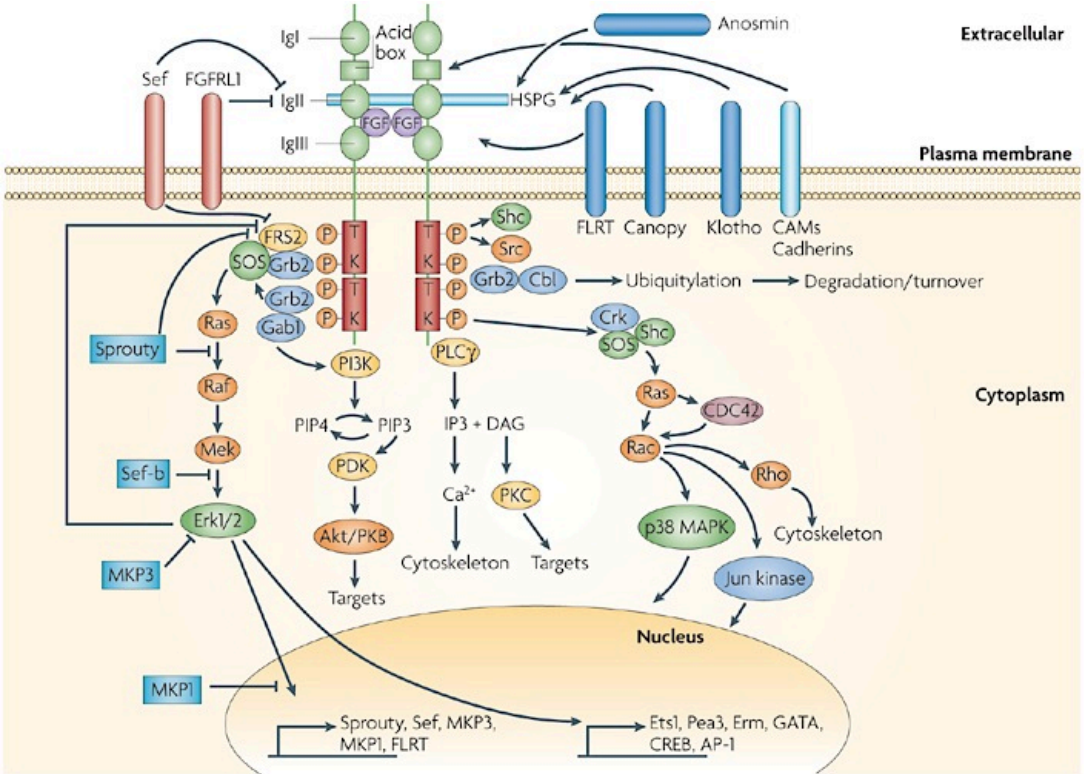


Figure 14: Various downstream effectors and positive and negative regulators of FGF signaling: Diagram illustrating the multiplicity of FGF signaling pathway that are activated downstream of FGFRs, together with the endogenous agonists and antagonists act both upstream and downstream of the receptor. FGF signals through Ras/MAPK, PI3K/Akt and PLC-γ pathways. (Adapted from [598])

Fibroblast receptor substrate 2:

One of the key differences between FGF signaling and other tyrosine kinase signaling is its immediate downstream effector FRS2. FRS2 (also known as suc1-asociated neurotropic factor target (SNT-1)) acts as control center for intracellular signaling initiated in response to FGF [599]. FRS2 is a downstream docking protein only for certain types of RTKs including FGFR, neurotrophin receptors (TrkA, TrkB, TrkC), ephrin receptors (Eph) (EphA4), RET and anaplastic lymphoma kinase (ALK). In contrast, FRS2 proteins do not transduce the signals for other RTKs like IGF, PDGFR or c-Met [600]. FRS2 is directly bound to the specific RTKs for which acts a docking protein via its PTB domain and becomes phosphorylated on tyrosine residues upon activation of the RTKs [601].

FRS2 exists in two forms in mammals namely FRS2α /SNT-1 and FRS2β/FRS3/SNT-2. The α form of FRS2 controls FGFR signaling whereas FRS2β negatively regulates EGFR signaling.

FRS2 domain structure: FRS2α and FRS2β share a similar structure. The N-terminus end is composed of the residues MGSCCS, which is a consensus myristylation sequence (MGXXXS/T) necessary for plasma membrane targeting. In both FRS2α and FRS2β, the C-terminus is made up of PTB domain with multiple tyrosine phosphorylation sites. FRS2α and FRS2β have 6 and 5 tyrosine phosphorylation sites respectively [602]. There is 72% homology among the PTB domains and the residues surrounding each tyrosine

phosphorylation site are also similar. The similarity of the residues surrounding the PTB is not as high as the PTB domain itself (Figure 15A) [603].

FRS2α-FGFR interactions: FRS2α interacts with FGFRs via the C-terminal PTB domain, which is independent of tyrosine phosphorylation. FRS2α binds to the juxtamembrane domain to non-phosphorylated peptides of the FGFR. This interaction between the FRS2-PTB domain and the FGFR is constitutive and independent of ligand binding and receptor activation (Figure 15B) [604]. This is opposed to the binding between the PTB domain of FRS2α and TrkA, TrkB or RET, which is mediated by tyrosine phosphorylated peptides that possess a NPXY motif in the receptors. Unlike, FGFR-FRS2α interaction, FRS2α-TrkA or RET interaction is thus dependent on the activation and phosphorylation of Y490 and Y1062 of the receptors, respectively [605].

FGFR-FRS2 interaction is more than just binding due to the alternative splicing of the juxtamembrane region. The alternative splice products of the juxtamembrane-coding region generate two isoforms with or without the two amino acids valine and threonine (VT). The VT sequence is major determinant to actuate FRS2α-FGFR1 interaction [606]. With respect to FGFR-IIIb, the residue Y770 is important for the regulation of FRS2α and its loss causes persistent activation and increased binding of FRS2α. It is noteworthy that FRS2α-FGFR1 and FRS2-TrkA interactions are thermodynamically different. The binding of FRS2 to Trk is largely enthalpy-driven, while the FGFR interaction is governed by a favorable entropic contribution of free energy of binding [607].

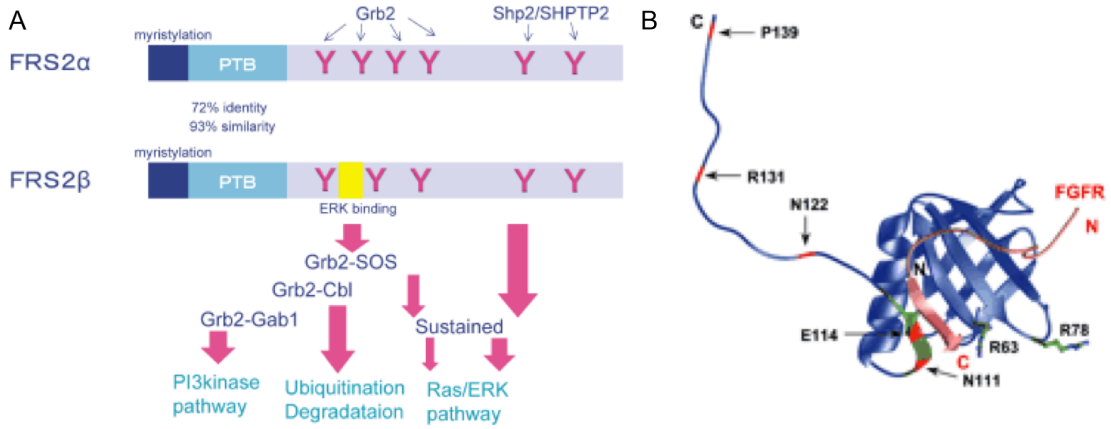


Figure 15: (A) Diagram showing the general domain structure of FRS2α and FRS2β (Adapted from [599]). **(B)** Ribbon representation of structure of PTB domain of FRS2α and its interaction with FGFR1. (Adapted from [608])

Molecular role of FRS2: FRS2α assembles both positive and negative signaling proteins to mediate a highly controlled and balanced transduction of signal via Grb2 and Shp2 leading to sustained ERK activation [602, 605]. FRS2α is involved in the negative regulation of FGF signaling by modulating SPRY proteins and ERK. ERK activated by FGF signaling phosphorylates FRS2α at multiple threonine residues rendering the inactivation of FRS2α and FGF signaling. There are eight canonical ERK phosphorylation sites (PXTP) in FRS2α, which are responsible for the negative feedback mechanism to be exerted on FGF signaling (Figure 16a). Expression of mutant FRS2α devoid of the ERK phosphorylation sites enhanced tyrosine phosphorylation of FRS2α, ERK activation, cell migration and cell proliferation [609].

FRS2α also acts a molecular hub for cross talk between FGF signaling and other growth factors signaling. ERK, which is activated by FRS2 independent mechanisms via EGF, PDGF or insulin signaling, phosphorylates threonine residues on FRS2α, thus contributing to the negative regulation of FGF signaling (Figure 16b) [610].

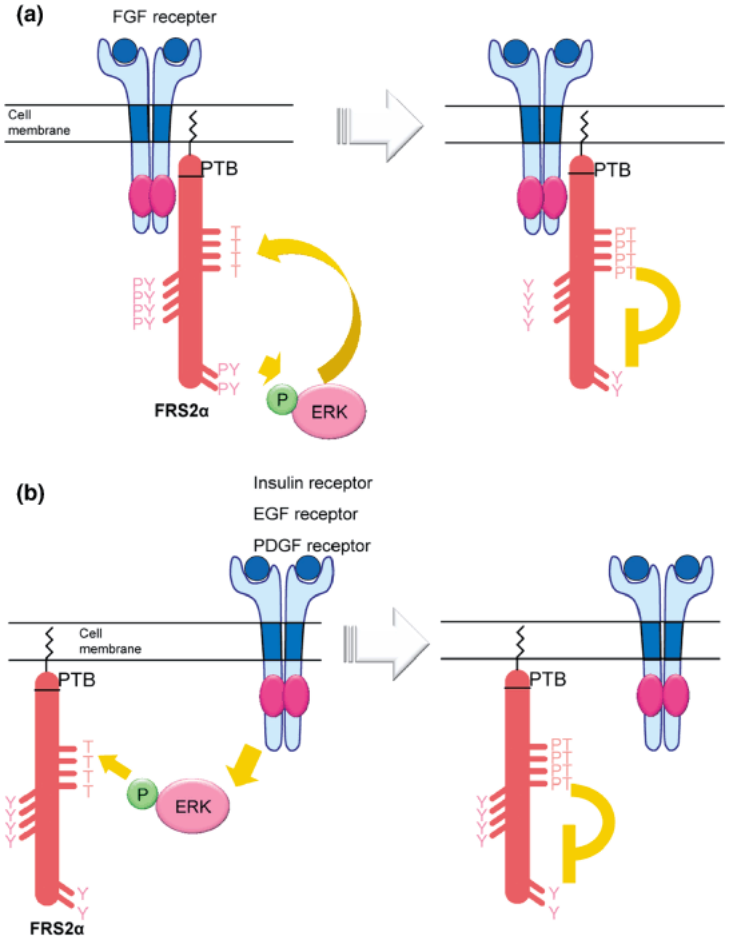


Figure 16: Negative regulation of FGF signaling via FRS2: (a) Activated FGF receptor phosphorylates tyrosine residues on FRS2α. The resultant activated ERK in turn phosphorylates threonine residues on FRS2α and inhibits tyrosine phosphorylation of FRS2α through a negative feedback loop. **(b)** Activated RTK induce ERK activation via FRS2 independent mechanisms. The activated ERK phosphorylates threonine residues on FRS2α and inhibits tyrosine phosphorylation of FRS2α. (Adapted from [599])

Physiological role of FRS2: The FRS2 gene is ubiquitously expressed during development and expressed of FRS2α is perceptible at embryonic day (E5) in mouse embryos. FRS2α knockout mouse models demonstrated that FRS2α plays pivotal roles in multiple developmental processes. Embryos lacking FRS2α have defects in the anterior-posterior axis formation. Lack of FRS2α disturbs the FGF-dependent maintenance of trophoblast stem cells resulting in developmental retardation and embryonic lethality [611]. Knockdown of FRS2α in adult mice did not have gross morphological defects except or eyelid developmental defects, which arise as a result of low penetrance [612]. Neural progenitor cells without FRS2α showed reduced proliferation upon FGF stimulation. Furthermore, specific ablations of FRS2α inhibited prostatic branching morphogenesis and growth in the prostrate epithelial cells [613].

FRS2α in cancer: Aberrant FGF signaling due to overexpression of FGFRs or FGFs is implicated in various cancers. In line with the deregulated FGFR signaling, one can conclude that the control center of FGF signaling (i.e.) FRS2α may also contribute to cancer. Till date, no specific role of FRS2α has been elucidated in cancers. However, FRS2 is oncogenic and found amplified in prostate cancer [614] and high-grade serous ovarian cancer [615] suggesting that it might contribute to enhanced FGFR signaling. Mostly, overexpression of FGFRs indirectly enhances the activation of FRS2α and ERK and the significance of FRS2α overexpression remains elusive.

Crosstalk between FGF and other cytokine signaling pathways:

As FGF is pivotal in the developmental stages, most of its crosstalk with other pathways is mostly evident during the developmental processes. In early mouse embryos, FGFs promote limb-bud outgrowth, which is inhibited by BMP. Throughout the developmental processes, the balance of signaling between BMPs and FGFs determines the rate of chondrocyte proliferation. Interestingly, the interaction between BMP and FGF pathways can either be positive or negative depending on the context. FGF signaling can antagonize canonical SMAD signaling while it can also have positive effects on Msx2 promoter via its non-ERK/MAPK downstream effectors [616]. FGF signaling has also shown to extend its interplay with SHH signaling. SHH initiates the expression of FGF4 in the ectoderm and it is positively regulated by a positive feedback loop. FGF acts upstream of the Indian hedgehog (Ihh) pathway and promote hypertrophic differentiation by directly influencing Ihh expression [617]. Similarly, IGF-1 prevents apoptosis induced by mutations in FGFR3 through the PI3K and MAPK pathways. Furthermore, Wnt signaling is involved in the FGF10 and FGF8 signaling loops in mesenchyme cells [618].

Very few studies have reported FGF signaling crosstalk pertinent to cancer. The FGF signaling is shown to interconnect with WNT signaling pathway in human colorectal cancers, mouse mammary virus induced tumours and E2A-pbx induced leukemia [619]. FGF signaling communicated closely with integrin pathway by directly binding to the αvβ3 integrin and resulting in the formation of FGF-FGFR1-integrin ternary complex. This signaling pathway fusion product is a potential therapeutic target in cancer, as obstructing this ternary complex can simultaneously shut down FGF and integrin pathways aberrantly activated in human cancers [620]. Similarly, FGF and EGF signaling interact with each other via FRS2, which acts a molecular switch and convergence point in PC12 cells [584].

These types of crosstalk prevalent in cancer cells highlights the need for the development of cocktail therapy with inhibitors, small molecules or neutralizing antibodies targeting all the pathways involved in the crosstalk.

1.8.5 Functions of FGFR signaling

The functions of FGFR signaling depend on the specific binding interplay between FGFs and FGFRs described above and affect the following biological processes:

Cell proliferation: FGFs induces cell proliferation in many cell types and the predominant ones being the endothelial cells, stem cells and epithelial cells. FGF1 is a strong mitogeneic factor for human pre-adipocytes and regulates the overall adipogenesis [621]. Analogous to FGF1, FGF2 is pro-proliferative and stimulates proliferation and survival of neuroepithelial cells isolated from the telencephalon and mesencephalon of p10 (10 days post-natal) mice [622]. FGF4 is an absolute necessity for trophoblast proliferation, as FGF4 knockout mice embryos die post implantation [623]. FGF7 is associated with epithelial cell growth. Interestingly, FGFs stimulate proliferation of both cancer cells and normal cells [624]. FGF10 facilitates the prostrate epithelial cell growth and proliferation, thereby contributing to the pathogenesis of prostrate cancer [625]. Furthermore, FGF18 triggers the proliferation of primary osteoblasts, prechondrocytic ATDC5 cells by inhibiting the differentiation and matrix synthesis of these cells [626].

Cell migration: Cell migration induced by FGFs varies with subfamilies. FGF1 and FGF2 play crucial roles in the migration of cochlear ganglion neurons in mice [627, 628]. FGF7 is known to stimulate the migration of human keratinocytes and its plasminogen activity [629]. In line with FGF2, the potent chemoattractant FGF8 promotes mesencephalic neural crest cells [630].

Cell differentiation: Cellular differentiation affects the size, shape, membrane potential, metabolic activity and responsiveness of a cell to signals, in which FGF signaling plays a central role. FGF-induced cell differentiation varies with FGF subfamilies. Similar to FGF1 and FGF2's role in migration, they also play an important role in the initial differentiation of cochlear ganglion neurons in mice. Moreover, FGF2 influences differentiation of neuroepithelial cells into mature neurons and gila [631]. As predicted, in addition to its role in the migration of keratinocytes, FGF7 differentiates suprabasal keratinocytes to mature keratinocytes [632]. Exogenous FGF20 is essential for the morphogenesis of stem cells to dopaminergic neurons in monkeys [633].

Angiogenesis: FGF signaling is less prominent in inducing angiogenesis as compared to other potent promoters of angiogenesis such as VEGF and PDGF. Only a limited number of FGFs among the 22 known FGFs have been tested for their angiogenic potential *in vitro* and *in vivo*. FGF1 and FGF2 indirectly contribute to angiogenesis by organizing the endothelial cells into tube-like structures [634].

1.8.6 FGFs and FGFRs in the brain

Expression and distribution of FGFs and FGFRs in the brain:

The elaborate signaling system of FGFs and their receptors partake in many developmental and repair processes of virtually all mammalian tissues. Ten out of the 23 FGF members are identified in the brain [635]. In the CNS, the most predominant FGFs are FGF1 and FGF2 with differences in cellular localization and distribution. Both neuronal and non-neuronal cells express FGF2, while FGF-1 is localized majorly in neurons [636].

In the developed CNS, FGF1 is ascertained in neurons of the oculomotor nucleus, the pons, the lateral geniculate nucleus, the reticular formation, the ventral tegmental area, the substantia nigra, the hypothalamus, the thalamus, the medial septum, cerebral cortex and the hippocampus. High levels of FGF1 mRNA are also present in the cerebellum, the locus coerules and the neocortex [637].

FGF2 is localized in neurons and glial cells. A wide distribution of FGF2 is found throughout the CNS and has been detected in the medulla oblongata, the pons, the colliculi, the thalamus, the olfactory bulb and cerebral cortex. Furthermore, neurons of the cortex, the hippocampus, the substantia nigra, brain stem, motor and sensory nuclei, anterior lobes of the pituitary also express FGF2 [638-640].

With regard to the FGF receptors, all FGFRs are expressed in the adults CNS. FGFR1-3 is highly expressed in the diencephalon and telencephalon while it is moderately expressed in mesencephalon and metencephalon. FGF expression is relatively negligible in the myelencephalon. FGFR1 is most widespread in neurons and is identified in neuronal populations, astrocytes and white matter tracts of adult CNS, while FGFR2 is predominantly found in glial cells [641]. FGFR4 expression is limited to the early stages of development and rarely detected in adult CNS [642].

Source of FGFs in the brain:

Glial cells are an important source for the synthesis and release of FGFs in the CNS. FGF2, the most predominant FGF in the CNS is prominently synthesized and released by the astrocytes. FGF2 released by astrocytes is imperative for growth of neuronal cell processes. Thus, the length of axons and dendrites of cortical neurons is increased when cultured with astrocyte-conditioned medium as compared to the culture with conditioned medium from fibroblasts. This was further made evident when neutralizing antibodies against FGF2 significantly mitigated the astrocyte dependent increase in the neuronal process growth. In addition, the astroglial release of FGF2 is also important for neuron survival and neurite outgrowth. The neurotransmitter dopamine acts as a enhancer of astroglial FGF2 release thereby promoting survival and process formation of tyrosine hydroxylase-positive neurons in E14 embryonic midbrain cultures [643].

Apart from FGF2, FGF9 is also secreted by the glial cells and are detected in specific populations of GFAP-positive astrocytes in the white matter tracks of the spinal cord and in the brain stem [644].

Functional role of FGF2 in the brain:

FGF2 being the predominant FGF in the CNS, it is quite predictable that it tunes diverse functions in the CNS. The most notable functions include areas of developmental patterning, neurogenesis, axonal growth, neuroprotection, lesion repair and learning and memory. These functions of FGF2 are elaborated below:

Neurogenesis and differentiation: The effects of FGFs on neuronal differentiation largely depend on the developmental stage at which the factor is applied. Exposure to FGF2 in early developmental processes extends the period of dopamine precursor division and in turn delays the differentiation [645]. FGF2 not only affects the proliferation of dopaminergic neurons but also affects the proliferation of GABAergic neurons. In both cell types addition of FGF2 in late development time point does not stimulate proliferation because transitions from a multi-potential to a postmitotic cell causes restrictions in neuronal fate. Thus FGF2 is selectively mitogenic only for embryonic spinal cord cells that are expressing neuronal phenotypes [646]. The proliferation fate of striatal stem cells is also governed by FGF2 [647]. Moreover, FGF2 is pro-proliferative for stem cells in hippocampus and serves as a differentiation factor for calbindin-expressing hippocampal neurons. Although, proliferation of neural stem cells and differentiation into mature neurons occurs predominantly in the early developmental stages, neural proliferation can also be perceived in adult brain. Proliferation of adult neuron precursor cells occurs in the hippocampus and sub-ventricular zone of forebrain and FGF2 regulates this adult neurogenesis. Identical to the hippocampus zone, cells of the striatum are able to proliferate and differentiate into astrocytes under the influence of FGF2 [648].

Axon growth and branching: Alongside neuronal precursor proliferation and differentiation, FGFs can alter neuronal morphogenesis. One of the characteristic features of the neuronal morphological differentiation process is the elongation and branching of the neuronal processes. While there are many factors like Brain derived neurotrophic factor (BDNF) and Neurotrophic -4 (NT-4) are known to influence axon branching, FGF2 is the most effective of all the regulators [649]. In the hippocampus, FGF2 selectively promotes bifurcation and growth of axonal branches resulting in complex axonal tress while it maintains a constant elongation rate of primary axons. This accelerated axonal branching is a specific attribute of FGF2 as removal of FGF2 ceases axonal branching. Axonal branch formation also requires a continuous presence of FGF2 for prolonged effectiveness [650, 651].

Neuroprotection and lesion repair: In vitro studies and brain lesion investigations have identified FGF2 as a potent trophic factor for many different populations of neurons. In the hippocampus, glutamate induced cell death is reduced by FGF2 by regulating the glutamate receptor subunits [652]. FGF2 does not exhibit the neuroprotective effects individually and it requires the presence of additional growth factors like glial cell line derived neurotrophic factor. FGF2 also extends its neurotropic effects on septal cholinergic, non-cholinergic neurons and mesencephalic dopaminergic neurons [653]. In line with neuroprotective properties of FGF2, it is natural that FGF2 also plays a possible role in brain lesion repair. FGF2 is able to prevent death of cholinergic and dopaminergic neurons after chemical injury. In accordance with these findings, brain lesions are often found with upregulated FGF2. Cortical lesions up-regulate FGF2 mRNA and protein levels for up to 2 weeks post insult, taking advantage of microglia and astrocytes as the primary source of FGF2 synthesis [654].

Ischemia: Destruction of distinct brain regions is a result of ischemic insults, which depends on the type of vascular occlusion applied to the brain. Hippocampal ischemic insults can be circumvented by the neuroprotective FGFs like FGF2, FGF7 and FGF1 thereby preventing the

rapid neuronal cell death [655-657]. Concomitantly, FGF2 levels are upregulated within the lesioned ischemic brain regions and endogenously synthesized FGF2 is necessary for the re-activation of proliferation and differentiation of neural progenitor cells after brain insult. The exact neuroprotective mechanism of FGF2 is not known. However, it is speculated that FGF2's ability to attenuate oxidative damage may point to its neuroprotective capacity [658, 659].

Learning and memory: Recent studies show that additional to FGF2's role in neuroprotection and neurogenesis, FGF2 is also important for adult brain cognitive processes like learning and memory [660].

Other significant FGFs in the brain:

Besides FGF1 and FGF2, FGFs like FGF3, 10, 7, 8, 9 and 18 are also significantly expressed in various regions of the developing and adult brain and perform various physiologically important roles. The interactions between unique combinations of brain-expressed FGF and FGFR isoforms lead to a whole array of functions including primary neural induction, neural precursor proliferation, pattering, neuronal specification and neurotrophism [661].

1.8.7 FGF signaling in cancer and tumour microenvironment

FGF signaling is exemplary for the multitude of physiological functions that are performed and regulated by FGFs and FGFRs. Due to its versatile role during development and for normal physiology; aberrations in FGF signaling are directly attributed to the pathogenesis of multiple types of cancer. These aberrations span a wide range including ligand-independent receptor signaling, alterations that support ligand-dependent activation, over expression and chromosomal translocations of both ligand and receptor [662].

Deregulation of FGFs and FGFs in cancer:

The mechanisms of deregulation of FGF signaling can be broadly based on the following mechanisms listed and explained below:

- *FGFR amplification or overexpression:* Deregulated transcription or chromosomal amplification of FGFRs induces cellular transformation of non-transformed cells. Amplifications of FGFR1 or FGFR2 are the most common FGF signal aberrations and have been reported in many cancers [26]. FGFR amplification is directly proportional to poor prognosis and approximately 10% of gastric cancers exhibit FGFR2 amplification [663]. FGFR amplification is implicated in enhanced cellular proliferation. FGFR1 overexpression has been predominantly observed in estrogen receptor-positive breast cancer, oral squamous cell carcinoma and ovarian cancer [664-666]. However, unlike FGFR2, FGFR1 over expression may not directly cause cancer cell proliferation [662].
- *Activating mutations:* A screen of more than 1000 somatic mutations in the coding regions of 518 protein kinases from a pool of 210 different human cancers revealed

that the FGF signaling pathways are the most commonly mutated with FGFR3 as the most frequently mutated FGFR [26]. Unlike mutations in the kinase domain in other RTKs, mutations in FGFR3 occur as a single point mutation at the extracellular domain. Roughly 60% of bladder cancers harbor mutations in FGFR3 [667]. Furthermore, cervical cancers, multiple myeloma and prostate cancer also have detectable levels of FGFR3 mutations [668-671]. In addition, FGFR2, which are also frequently mutated in the extracellular domain are found in endometrial cancers [672]. Mutations in FGFRs can also render the receptors insensitive to endocytosis by maintaining their expression at the cell surface [673].

- *Autocrine and paracrine signaling:* Gene amplifications and mutations in FGFR lead to a constitute activation of the receptors independent of ligand binding. Apart from this mechanism, ligand-dependent signaling can also drive pathogenesis of cancer via either autocrine production of ligands in cancer cells or paracrine production of ligand from stromal cells. FGF can promote cancer by both autocrine and paracrine signaling as ectopic expression of FGF in either epithelial cells or stromal fibroblasts stimulated cancer cell proliferation [674]. FGF2-FGFR1 autocrine loop promotes development of melanoma. In prostate cancer, several FGFs including FGF2 are upregulated, suggesting the potential existence of a paracrine loop [675]. Similarly, increased levels of endocrinal FGFs such as FGF19 are elevated in hepatocellular carcinomas, a subgroup of liver, colonic and lung squamous carcinoma reflecting an autocrine loop dependent tumorigenesis [676-678].
- *Germline single nucleotide polymorphisms:* Genome wide association studies identified that a SNP located in the second intron of FGFR2 correlated with increased risk of developing breast cancer [679]. Likewise, a second SNP in FGFR4 is associated with poor prognosis in breast, colon and lung carcinomas but it does not seem to increase the incidence of cancer [680, 681].
- *Chromosomal translocations:* The link between FGFRs and oncogenesis is best studied via chromosomal aberrations. 15% of multiple myelomas have a (4;14) translocation between FGFR3 and Ig heavy chain [682, 683]. This translocation causes abnormal ligand-dependent signaling and correlates positively with poor prognosis.

Oncogenic mechanisms of FGF signaling:

FGF signaling promotes tumorigenesis by affecting a range of its downstream biological processes as elaborated below:

- *FGF and proliferation:* Activating mutations, chromosomal translocation and overexpression of FGFs/FGFRs promotes cancer cell proliferation of tumour cells. In hematological malignancies, zinc-finger 198-FGFR1-fusion protein deletes the FRS2 binding site of FGFR1 and activates STAT5 for tumour cell proliferation [684, 685]. Analogously, breakpoint cluster region (BCR)-FGFR1 fusion proteins in myelogenous leukemia activate Grb2, thus promoting FGFR1-induced proliferation [686]. In prostate cancer, overexpression of FGF10 leads to the activation of PI3K/AKT pathways and promotes enhanced cell division in the prostate epithelium [687].

- *FGF and survival:* FGF signaling actuates survival by activating anti-apoptotic pathways via the activation of PI3K/AKT or STAT signaling. This pro-survival function of FGF is a key reason for the development of resistance to chemotherapy [688]. In addition to the activation of PI3K and STAT pathways, FGF2 can also up-regulate the expression of anti-apoptotic proteins like BCL-2, to mediate its cytoprotective effects [686]. Moreover, in cancerous urothelial cells, FGF2-induced proliferation and decreased apoptosis is achieved by the MAPK signaling through FRS2 [689].
- *FGF and migration and invasion:* FGFR1 induced invasion is evident via the activation of MAPK and PI3K pathways in breast cancer models [690]. EMT is necessary for invasion and migration. The EMT induced, FGF target gene SOX9 is upregulated in prostate carcinomas indicating the potential role of FGFs in cancer cell invasion and metastasis [691]. Surprisingly, FGF signaling can also promote invasion and migration through the non-classical pathways by interacting with the chemokine receptors CXCR4b and CXCR7b, which are responsible for coordination of collective migration during morphogenesis [692].
- *FGF in angiogenesis:* FGF1 and FGF2 act as potent pro-angiogenic factors in endothelial cells expressing high levels of FGFR1-IIIc and FGFR2-IIIc. Most of the pro-angiogenic properties of FGF2 are transduced through MAPK pathways [693]. However, activation of PKC is also required for endothelial cell proliferation and migration [694]. FGF2 immobilized by endothelial cells binds to αvβ3 integrin, causing endothelial cell adhesion and morphogenesis [693].

Dual role of FGFs: FGF tumour suppressive effects in cancer:

Abundant FGFs and FGFRs in the tumour microenvironment not only promote carcinogenesis but can also suppress tumour progression in certain contexts. Specifically, in MB, FGF in the tumour microenvironment inhibits SHH signaling, which blocks the proliferation of MB cells [695]. Similarly, FGFR2 is found to impede proliferation in bladder cancer, prostate cancer and salivary adenocarcinomas. Surprisingly, in prostate cancer, expression of a conditionally active form of FGFR1 promoted proliferation but the active form of FGFR2 did not [696-698].

Thus, FGFR signaling is generally pro-oncogenic but its potential tumour suppressive effects could be pointed towards the context-dependent signaling of FGFs that may either promote or regress the tumour. FGF signaling are speculated to induce cytoprotective effects by activating pathways that help to maintain genomic stability following challenges with carcinogens, reactive oxygen species and other cytotoxic stress [662]. FGFR2 may also have a potential role in immune surveillance, which may explain its tumour suppressive behavior. The ligands of FGFR2 namely FGF7 and FGF10 are released by Υδ-T cells to promote immune surveillance in epithelial cells and loss/mutations in FGFR2 can promote tumour progression [699].

1.8.8 Targeting FGFR signaling in cancer

FGFR-targeted therapeutics using antibodies and small molecule inhibitors are a buzz-word in the field of clinical cancer research because targeting FGF signaling can bring the various hallmarks of cancer such as proliferation, evasion of apoptosis, angiogenesis, EMT and invasion under control [700].

Small molecules tyrosine kinase inhibitors targeting FGFRs:

Non-selective FGFR TKIs: Small-molecules designed to fit the ATP-binding pocket of tyrosine kinase domains of the RTKs in an ATP competitive or ATP non-competitive manner. Due to the structural homology between the tyrosine kinase domains of various RTKs, non-selective TKIs like ponatinib, ninetedanib, dovitinib and lucitanib are capable of inhibiting FGFR signaling. As these compounds simultaneously target VEGFR, PDGFR and FGFR signaling, these compounds are mostly developed and referred to as 'anti-angiogenic' agents. Although concurrent inhibition of multiple RTKs enhances treatment efficacy by inhibiting redundant pathways, it also increases the treatment-related side effects. This calls for the development of more specific FGFR inhibitors [700]. In brief, Lucitanib inhibits VEGFR-1, PDGFR-α/β and FGFR1 and showed impressive efficacy in phase I/II trail for breast cancers. Phase II trials are ongoing for metastatic breast cancer and FGFR1-amplified squamous non-small cell lung cancer [701]. Dovitinib exhibits its effect against VEGFR1, PDGFR, FGFR1 and 3, FLT-1 and TrkA and Ponatinib is a multifunctional inhibitor capable of inhibiting BCR-ABL, LYN, FGFR1-2, VEGFR2 and PDGFR-α. FDA approved Ponatinib for the treatment of chronic myeloid leukemia [702, 703]. Other non-selective TKI, which inhibit FGFR but also target other kinases are brivanib, lenvatinib and orantinibv [662].

Selective FGFR TKIs: JNJ-42756493 (Johnson & Johnson) is a potent pan-FGFR inhibitor that can be administered orally. Phase I studied are ongoing in patients with FGFR3-TACC3 fusions in bladder cancer and glioblastoma [704, 705]. Similarly, AZD4547 (AstraZeneca) is a highly selective FGFR1-3 inhibitor, currently in phase II trials for gastric/esophagogastric cancers harboring FGFR1 amplification [706]. Another selective FGFR1-3 inhibitor, BGJ398 (Novartis) is currently in phase II clinical trials [707, 708]. LY287445 and Debio 1347 are other potent selective FGFR TKIs that are being evaluated in phase I trails [703]. Beside aforementioned reversible FGFR TKIs, TAS-120 is a second-generation highly potent irreversible inhibitor in phase I trails [709].

Monoclonal Antibodies against FGFR:

The past decade has seen tremendous advances in immunotherapy against FGFR signaling. MFGR1877s (Genentech) is a human anti-FGFR3 monoclonal antibody (mAb) that showed antitumor activity in preclinical models of bladder cancer with FGFR3 amplification and it is in Phase I trails for the same [710].

FGF traps:

FGF traps are a unique in-direct way of inhibiting FGFR signaling. FGF ligand traps sequester of FGF ligands, thereby making them unavailable for FGFRs. FP-1039 (GSK3052230, GlaxoSmithKline) is a soluble fusion protein made up of the extracellular domain of FGFR1c coupled to the Fc region of IgG1, which prevents binding of FGF1, FGF2 and FGF4. A phase II trail with FP-1039 was withdrawn because of unfeasible protocols. However, there is an ongoing phase I trail as a combination therapy with chemotherapy in patients with lung cancer [711].

The various strategies to target FGF signaling and the different TKIs, mAb and FGF traps in the clinical trails are summarized in the figure 17.

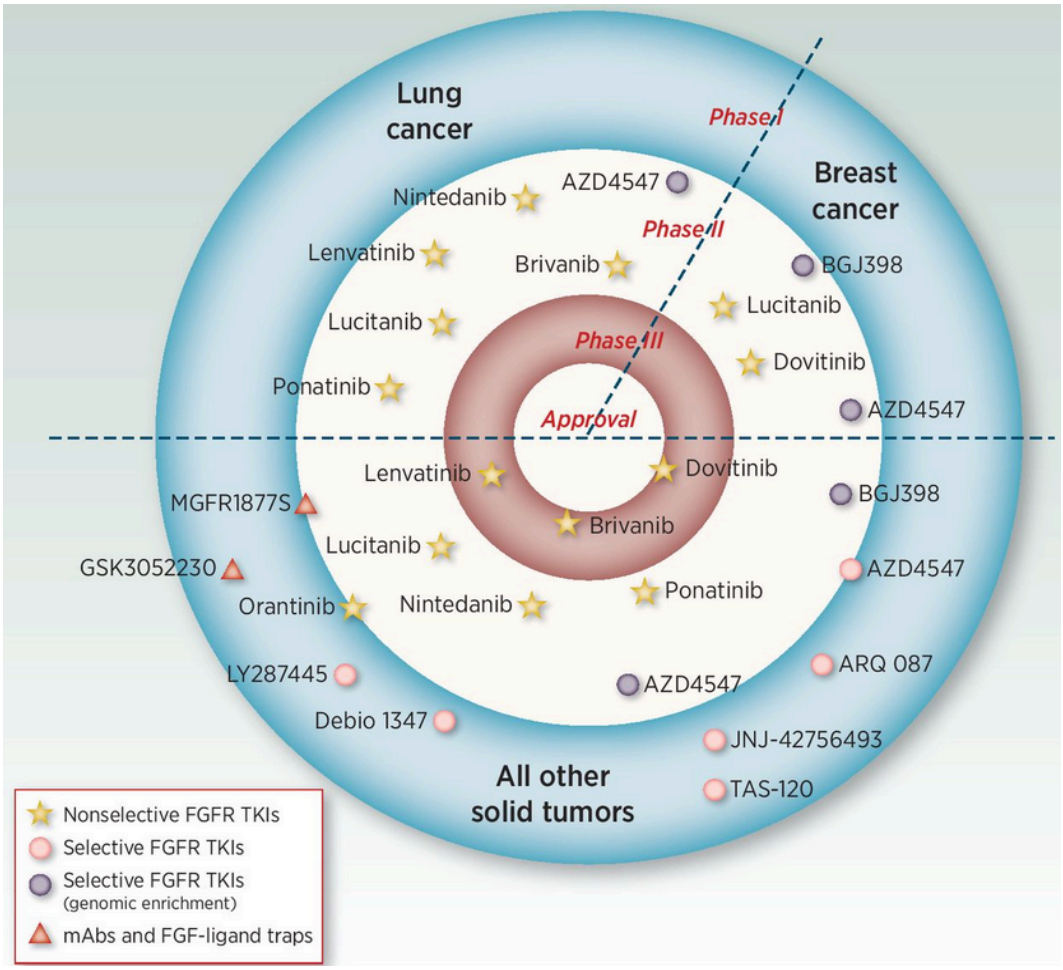


Figure 17: Strategies to target FGF signaling and clinical trials of putative FGF selective and non-selective FGFR inhibitors. (Adapted from [700])

1.9 Transforming growth factor receptor signaling

Transforming growth factor receptor (TGFR) signaling is one of the best studies signaling networks since its discovery in 1970s in various organisms from simple metazoans to humans. Spanning over 30 identified members, TGF-β superfamily of secreted factors are ubiquitously expressed in diverse tissues and function during the earliest stages of development and throughout the lifetime of animals.

1.9.1 Types of transforming growth factor-β

Transforming growth factor-β:

Transforming growth factor-β (TGF-β) belongs to the TGF-β superfamily, which is comprised of a large group of proteins, including the activin/inhibin family, bone morphogenetic proteins (BMPs), growth differentiation factors (GDFs) and the glial cell line derived neurotropic factor (GDNF) [712].

Having been discovered in a variety of species including vertebrates and invertebrates, TGF-β subfamily is a fundamental regulator of innumerable biological processes such as growth, development, tissue homeostasis and regulation of immune system [713]. TGF-β subfamily growth factors are homodimeric or heterodimeric polypeptide chains. The regulatory properties depend largely on cell type, growth conditions provided and presence of other polypeptide growth factors. The expression of TGF-β subfamily ligands is controlled by distinct promoters and its secretion is temporal and tissue specific [714].

There are three known highly conserved isoforms of the TGF-β subfamily expressed in mammalian tissues. The isoforms are TGF-β1, TGF-β2 and TGF-β3. They share highly conserved regions but differ in several aa regions. However, all isoforms function via the same receptor and signaling pathways [715, 716]. The three isoforms of TGF-β subfamily are explained below:

TGF-β1: TGF-β1 was first cloned from human term placental mRNA and it the most abundant and ubiquitously isoform of the TGF-β subfamily [717]. TGF-β plays an important role in growth and differentiation, which is highlighted by its expression, and localization in cartilages, endochondral and membrane bone and skin [718].

TGF-β2: TGF-β2 (also known as glioblastoma-derived T cell suppressor factor (G-TsF)) was first identified in human glioblastoma cells. As the name suggests, TGF-β2 suppresses interleukin-2 supported growth of T cells [719]. It shares a 71% homology with TGF-β1 [720] and unlike TGF-β1; TGF-β2 is particularly expressed only in neurons and astroglial cells in embryonic the nervous system. TGF-β2 is also implicated in enhancing tumour cell growth and proliferation either by autocrine signaling or by disrupting immune-surveillance [721].

TGF-β3: The third isoform, TGF-β3 shares an 80% homology with TGF-β1 and TGF-β2 and was first isolated from the cDNA library of a human rhabdomyosarcoma cell line. TGF-β3 is

localized and expressed in very high levels in umbilical cord [720]. TGF-β3 functions essentially in normal palate and lung morphogenesis and is involved in EMT transition [722, 723]. Consistent with its role in EMT, TGF-β3 mRNA is present in lung adenocarcinomas and kidney carcinoma cell lines.

1.9.2 Types of transforming growth factor β receptors

TGF-β receptors:

There are three types of receptors involved in TGF-β signaling: TGF-β receptor I (TβRI), TGF-β receptor II (TβRII) and TGF-β receptor III (TβRIII). TβRI and TβRII mediate signal transduction while TβRIII serves as a co-receptor. Both TβRI and TβRII are transmembrane serine / threonine kinases [716]. They are organized sequentially into the following domains:

- N-terminal extracellular binding domain
- Transmembrane domain
- C-terminal serine / threonine kinase domain

TβRI and TβRII are functional only in the tetrameric conformation, which they form via homo- or heteromeric associations. Type I receptors are smaller and their size ranges from 65 to 70 KDa as compared to the type II receptors, which range from 85 to 110 KDa. Although, smaller in size type I receptor possess a characteristic, highly conserved 30 aa Gly / Ser regulatory (GS) domain in the cytoplasmic domain [724]. The phosphorylation of the cytoplasmic domain of TβRI is a prerequisite for the complete activation of TβRII. TβRII has a 10bp polyadenine repeat, which codes for lysine in the coding region of the extracellular domain, which acts a binding pocket for TGF-β [725]. This binding pocket is often prone to frameshift missense mutations or early protein terminations that result in truncated or inactive receptor products [726].

TGF-β co-receptors:

TβRIII (also called betaglycan) is a cell-surface chondroitin sulfate / heparan sulphate proteoglycan, which is the largest, most abundant binding molecule (250-350 KDa) expressed in most cell types of fetal and adult tissues [727]. Together with betaglycan, Endoglin (CD105) also acts as TβRIII for TGF-β. In contrast to betaglycan, Endoglin is a RGD (integrin binding tripeptide composed of L-arginine, glycine and L-aspartic acid) containing glycoprotein that is expressed in a restricted set of cell types such as vascular endothelial cells, hemotopoetic cells, bone marrow stromal cells and chondrocytes [728]. The expression of endoglin is enhanced in active vascular endothelial cells during tumour angiogenesis in cancers like invasive breast cancer and renal cell carcinoma. Endoglin is also expressed in normal brain, adventitia and arterioles [729]. Due to the lack of the kinase domain, betaglycan and endoglin are not directly involved in intracellular TGF-β signaling. Instead, they act as co-receptors by controlling the accessibility of TGF-β to TGFRs thus indirectly modulating intracellular TGF-β activity [730, 731]. Betaglycan binds to all three isoforms of TGF-β with a constant affinity, while there is slightly higher affinity towards TGF-

β2. On the other hand, endoglin binds to TGF-β1 and TGF-β3 with high affinity and has a weak affinity for TGF-β2 [728, 732].

1.9.3 Mechanisms of TGF-β and TGFR activation

TGF-β synthesis and activation:

The three isoforms of TGF-β are synthesized as precursor proteins (390-412 aa) and require intracellular modification prior to secretion [733]. One of the important intracellular modifications is the cleavage (between aa 278 and 279) of the C-terminal pro-region from the N-terminal part of the protein by the furin endopeptidases [734]. The C-terminal pro-region is referred as latency-associated peptide (LAP) and the N-terminal region is the mature or active TGF-β [735]. However, even after the proteolysis from its pro-peptidases, LAP remains covalently associated with the mature N-terminal region [736]. This covalently LAP-mature N-terminal TGF-β complex is called as Latent-TGF-β (L-TGF-β) or large latent complexes (LLC). L-TGF-β cannot interact with TGFR and it has no known biological function. For TGF-β to exercise its biological function, it has to be converted from the L-TGF-β to active form [735]. There are two mechanism of activation of L-TGF-β.

1. TGF-β has to released from LAP
2. L-TGF-β has to undergo a conformational change so that it can bind to TGFR even when it is not released for LAP.

Since TGF-β is implicated in the pathogenesis of numerous diseases, the release and activation of TGF-β is one of the most important control and regulatory mechanism and it is largely context-dependent.

There are number of proteases that are capable of releasing TGF-β from LAP including serine proteases, plasmin, endoglycosidase F, sialidase, neuraminidase, cathepsins B and D, calpin and glycoproteins like thrombospondin-1 [733]. Among the aforementioned proteases, the plasminogen / plasmin proteolytic system is the most common mechanisms. Plasmin proteolytic system is unique as it acts as both positive and negative regulator of the release of TGF-β. The formation of plasmin from plasminogen via uPA allows localized proteolysis of L-TGF-β, thus releasing active TGF-β from LAP (Figure 18) [737]. However, active TGF-β is a potent inducer of PA1-1, which when released in the ECM degrades uPA, thus lowering the levels of plasmin [738]. The other non-proteolytic mechanism of TGF-β includes activation by reactive oxygen species, αvβ6 integrin and some intracellular parasite like *Leishamanias braziliensis*. The termination of active TGF-β synthesis via non-proteolytic mechanisms happens simply when the substance causing the activation is removed [739].

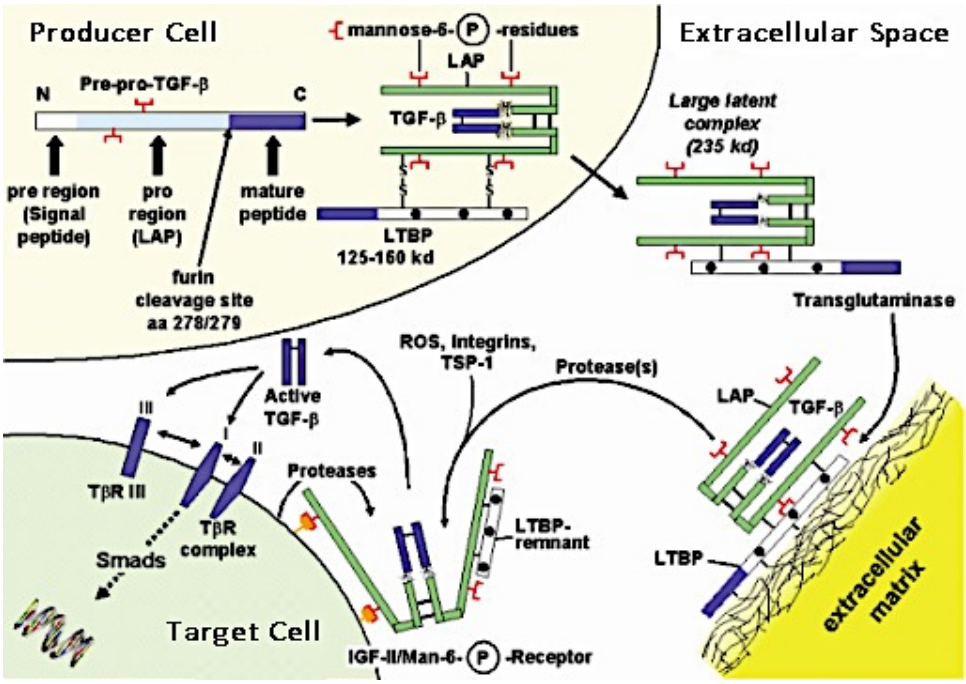


Figure 18: Synthesis and activation of TGF-β: Schematic representation of the various steps involved in the synthesis of pro-TGF-β and activation and release of functional TGF-β from L-TGF-β complex. (Adapted from [740])

TGF-β receptor activation:

Active TGF-β released from LAP is stable only as dimers. TGF-β dimers are formed and stabilized via hydrophobic interactions and interunit disulphide bonds. Stable TGF-β transduces intracellular signaling via bringing type I and type II receptor pairs together. TGF-β dimers induce TGF-β receptor pairs to form heterotetrameric receptor complexes [741]. Besides, tetrameric complex formation, binding of TGF-β to the extracellular domains of both receptors triggers the proper conformation of the intracellular kinase domains. The receptor complexes are then subjected to post-translational modifications (phosphorylation, ubiquitylation and sumoylation) whereby the receptors are stabilized and TGF-β induced SMAD and non-SMAD pathways are activated [742].

The phosphorylation of TGFRs activates TGF-β signaling pathway in the following phosphorylation cascade (Figure 19):

1. TGF-β binds to TβRII
2. TGF-β-TβRII complex phosphorylates the GS domain within TβRI.
3. Following phosphorylation of the GS domain, TβRI is incorporated in the signaling cascade, leading to the formation of a large ligand-receptor complex consisting of dimeric TGF-β ligand and two pairs of TβRI and TβRII (tetrameric conformation).

TGF-β1 and TGF-β3 are capable of binding to TβRII and activating the signaling cascade without the involvement of TβRI. In contrast, TGF-β2 interacts with TβRII only with the combination of TβRI. Ligand binding induces sufficient autophosphorylation of TβRII cytoplasmic domain and the signal is transduced without the presence and interaction of TβRI. TβRII enhances TGF-β signaling by binding to the appropriate TGF-β according to their

binding affinities. Although, endoglins always promotes TGF-β signaling, it may inhibit TGF-β in certain context and cell types. For example in chondrocytes, it enhances TGF-β1-induced SMAD1/5 phosphorylation but inhibits TGF-β1 induced SMAD2 phosphorylation, which is required for active TGF-β signaling [714].

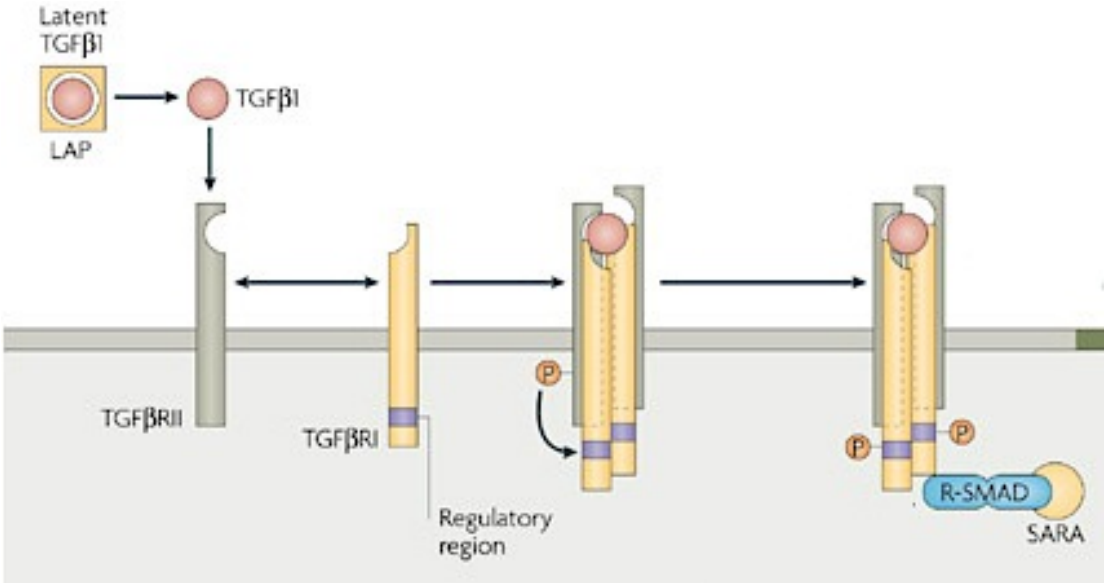


Figure 19: Activation of TGF-β receptor: Schematic representation of the cascade of activation of TGF-β receptor. Activated TGF-β binds to TβRII and the resultant complex in-turn binds and activates TβRI allowing the activation of downstream effectors of TGF-β signaling. (Adapted from [743])

1.9.4 Canonical pathways of TGFβR

Activation of TGF-β receptors may initiate both canonical (SMAD dependent) and non-canonical (non-SMAD) pathways of TGF-β (Figure 20). SMAD-dependent pathways are classical TGF-β signaling functions and majority of the studies have focused on SMAD dependent events [744]. SMA/MAD homology (SMADs) function as signal transducers of TGF-β superfamily. SMAD2 and SMAD3 serve as signal transducers for Activin, Nodal and TGF-β subfamily. The SMAD2/3 which are directly phosphorylated by the receptors are collectively referred to as 'receptor-phosphorylated SMADs (R-SMADs) [745]. Upon receptor-mediated phosphorylation of R-SMADs at the C-terminal serine residues, it recruits SMAD4 with high affinity. As SMAD4 function as a shared partner of R-SMAD, it is also called as co-SMAD. Co-SAMDs are required for the assembly of active transcriptional complexes. In addition to SMAD4 association, R-SMADS also binds to transcriptional co-activators such as p300 and CREB binding proteins (CBP) [746].

In the basal state (absence of TGF-β ligand), SMADs are retained in the cytoplasm. SMAD Anchor for Receptor Activation (SARA) facilitates the retention of R-SMADs, specifically SMAD2. In addition to restricting SMAD movements within the cells, SARA also obstructs a region of SMAD2 that mediates nuclear import. In the presence of TGF-β, receptor mediated phosphorylation enhances the association between SMAD2/3 and SMAD4 on one hand and decreases the affinity between SMAD2 and SARA on the other hand. This reduced affinity between SMAD2 and SARA causes the release of SMAD2, thus unmasking its nuclear import function leading to a rapid accumulation of the activated SMAD2 in the nucleus. SARA

contains a FYVE domain adjoining SMAD binding domain. FYVE domains facilitate the anchoring to endosome membrane [747]. This indicates that TGF-β receptor my undergo internalization upon activation.

SARA-inhibited nuclear transport of SMADs is independent of the nuclear localization signal (NLS) import pathway. The NLS pathway imports protein that contains a lysine and arginine-rich sequence known as NLS. Importin-α, the nuclear import factor recognizes the NLS as an extended peptide loop. SMAD2 harbors a lysine rich sequence, which when mutated impaired the nuclear transport of SMAD2. This suggests SMAD2 can be transported to the nucleus via the NLS pathway [748]. Interestingly, SMAD4 also has an intrinsic, agonist-independent nuclear export signal, which keeps SMAD4 in the cytosol [749]. Therefore, SMADs are functional nuclear proteins that are maintained in the cytoplasm so that they are accessible to the activated receptors at the plasma membrane [750].

1.9.5 Non-canonical pathways of TGFβR

TGF-β signaling occurs predominantly via SMAD signaling. However, the diverse functions of TGF-β family cannot be reconciled with a simple SMAD signaling model. Nowadays, it is well characterized that TGF-β also signals via non-SMAD components to exert its functions (Figure 20) [751]. The well known non-SMAD signaling pathways of TGF-β are listed and explained below:

TGF-β activated ERK / MAPK pathway:

TGF-β can rapidly activate p21 (Ras), thus providing the first indications of activating the ERK/MAPK pathway [752]. The rapid activation of Ras by TGF-β may cause the recruitment of Raf to the plasma membrane, subsequently leading to the activation of ERK via MEK1 [752, 753]. Indeed, rapid phosphorylation of ERK by TGF-β is observed in epithelial cells, fibroblasts and breast cancer cells [754, 755]. There are two different kinetics of ERK activation by TGF-β that varies and depends on the cell type and culture conditions. There is either a delayed response of ERK to TGF-β with the peak of phosphorylation occurring only after hours of stimulation or an instant activation within 5-10 minutes comparable to ERK activations by other mitogens. The delayed response of ERK by TGF-β, which may be due to SMAD-dependent transcriptional response, explains the indirect activation of ERK by TGF-β [756].

It is well known that a tyrosine-phosphorylated residue on the receptor is required for the docking of the cascade of adaptor proteins in the classical RTK signaling. Although, TβRI and TβRII are serine-threonine kinases, they can undergo autophosphorylation on three tyrosine residues namely: Y259, Y336 and Y424. This phosphorylation is much lower in levels than autophosphorylation of Ser/Thr residues but it is sufficient to serve as docking sites for the recruitment of Grb2 and Src, thereby bridging TβRII to MAPK activation. The tetrameric receptor heterocomplex nature of TGFRs makes it difficult to explain whether the tyrosine phosphorylation stems from autophosphorylation of TβRI or via transphosphorylation of TβRII [757]. Nevertheless, phosphorylated tertrameric TGFR complex leads to the formation

of ShcA/Grb2/Sos complex by directly phosphorylating and recruiting ShcA. The activated ShcA/Grb2/Sos complex then transduces the signal to Ras at the plasma membrane, resulting in a sequential activation of c-Raf, MEK and ERK. The rate-limiting step of this cascade is the phosphorylation of ShcA. Overexpression of a mutant lacking the PTB domain of ShcA or siRNA silencing of ShcA lead to abrogation of ERK phosphorylation, thus confirming the important role of ShcA in ERK activation by TGF-β. Even though ShcA is capable of being phosphorylated by both TβRI and TβRII, ShcA preferentially interacts with, and is phosphorylated more efficiently by TβRI as compared to TβRII [758].

ERK activation is the key element for EMT, which is one of the major biological functions of TGF-β. In the process of tumorigenesis, TGF-β promotes tumour progression by inducing EMT via both SMAD-dependent and SMAD-independent components [759, 760]. ERK activation is the non-SMAD pathway that is required for the disassembly of cell adherent junctions and induction of cell motility. TGF-β induced ERK activation exercises its role in EMT via controlling the transcription of the target genes responsible for matrix interaction, cell motility and endocytosis. Here again, phosphorylation of ShcA is required for TGF-β induced ERK activity and EMT. Abrogation of ShcA with siRNA renders tumour cells unresponsive to TGF-β induced EMT, highlighting that Shc-Grb2-ERK pathway is a strategic component of pro-oncogenic activities of TGF-β that is involved in invasiveness and metastasis [761]. In addition to the modulation of various target genes responsible for EMT, ERK can also regulate SMAD1, SMAD2 and SMAD3 and AP-1 family members in the context of TGF-β signaling [762].

TGF-β induced JNK/p38 pathway:

The best-characterized non-SMAD pathways of TGF-β are the JNK/p38 MAPK cascades. Like ERK activation, the activation of p38 and JNK by TGF-β is quite rapid. SMADs are dispensable for TGF-β-induced JNK activation as dominant negative forms of Smad3 or Smad4 deficient cells did not affect TGF-β induced JNK activation [763]. Analogues to the ERK, JNK and p38 are at the tertiary level of the MAPK cascade and rely on being activated by MAP kinase kinases (MKKs), specifically MKK4 and MKK3/6 respectively [764]. The upstream activators of MKKs are MAP3Ks, which are activated by TGF-β1 activated kinases (TAK1). TAK1 is shown to interact directly with the TβRII, but the exact mechanism of TGF-β activated TAK1 is unclear [765]. TNF receptor associated factor 6 (TRAF6), which is involved in the activation of TAK1 in interleukin-1 receptors (IL-1R) and Toll like receptor (TLRs)-mediated pathways, also plays a vital role in TGF-β induced TAK1 in JNK/p28 pathways [766]. Further upstream of TAK1, TRAF6 functions as a recruiter of TAK1 through its polyubiquitinated chains. Unlike lysine-48 (K48)-linked polyubiquitination, which normally tags the proteins for degradation, polyubiquitinated TRAF6 acts as scaffold to assemble protein kinase complexes and mediates activation of TAK1 [767]. Apart from activating TAK1, TRAF6 can also interact directly with TβRI and TβRII via its C terminal TRAF domains. This association does not require the kinase activity of TGFR, whereas polyubiquitnation of TRAF6 is facilitated by TGFRs. Sandwiched between TRAF6 and TAK1 are two other MAPKKKs namely MEKK1 and MLK3 that function upstream of TGF-β to activate JNK or p38 via MKK4 or MKK3/MKK6, respectively [768].

Although TGF-β induces JNK/p38 activation is independent of SMAD activation, the JNK/p38 pathway and SMAD pathways are not mutually exclusive. The TRAF6-TAK1-JNK/p38 pathway combines with the SMAD pathway to regulate the cellular response of TGF-β. This implicitly explains the role of JNK/p38 in TGF-β induced apoptosis, which is a well-recognized mechanism of the canonical TGF-β SMAD pathway [769]. In addition to apoptosis, TRAF6-TAK1-JNK/p38 cascade also plays an important role in TGF-β induced EMT. Inhibition of p38 activity, dominant negative forms of MKK3 or silencing TRAF6 impairs TGF-β mediated changes in cell shape and actin-reorganization required for EMT, thus highlighting the prominence of this pathway in TGF-β induced EMT [770].

Rho like GTPases induced by TGF-β:

A direct link between TGF-β and its role in promoting EMT is elucidated via its rapid activation of RhoA. Rho-like GTPases including RhoA, Rac and Cdc42 exhibit important roles in controlling the dynamic cytoskeletal organization, cell motility and gene expression. TGF-β-induced RhoA leads to the formation of stress fibers and mesenchymal characteristics requires for EMT [771, 772]. Similar to JNK/p38 pathway, RhoA activation of TGF-β is also independent of SMAD2/3 as dominant negative forms of SMAD3 were incapable of reducing TGF-β-induced RhoA activation. Although the SMAD pathway does not directly interfere with the activation of RhoA by TGF-β, it can influence the peak of RhoA activation via TGF-β induced NET1, a RhoA specific guanine exchange factor that mediates RhoA activation by SMAD dependent transcription [773].

Controversially, TGF-β can downregulate RhoA levels in certain contexts like dissolution of tight junctions, which is a prerequisite for EMT. TGF-β lowers the levels of RhoA via Par6 activation. Presence of TGF-β induces the assembly and accumulation of TβRI and TβRII complexes at the tight junctions, where it phosphorylated Par6. Following phosphorylation, Par6 recruits Smurf1 and the Par6-smurf1 exerts a localized ubiquitination and turnover of RhoA at the cellular protrusions, thereby dissolving the tight junctions [774]. The localized Smurf1-induced degradation of RhoA requires the presence of Smurf1 in cellular protrusions. In addition to the association with Smurf1, Par6 also associated with Cdc42/Rac1 to activate PKC-ζ to further facilitate the localization of Smurf1 and degradation of RhoA at the cellular protrusions. This notion is confirmed, as silencing of Smurf1 did not change the net RhoA protein levels, instead it lead to the accumulation of RhoA associated F actin in the cellular protrusions [775]. Collectively, TGF-β regulates RhoA via two different modes:

1. Rapid activation of RhoA during the early phase of stimulation
2. Down-regulation of RhoA at the later stages to promote EMT

Apart from RhoA, TGF-β can also activate Cdc42 GTPases, independent of the SMAD signaling pathway. Concomitantly, impeding the function of SMAD2/SMAD3 did not affect the levels of p21 activating kinase 1 (PAK1), which acts downstream of Cdc42. Cdc42 directly interacts with TGFRs and triggers the activation of the following PAK mediated cascade: the

Rac1 exchange factors α-PIX and β-PIX, PAK1, PAK1-interacting partners, oxidative stress responsive kinase-1 and occludin (OCLN), a tight junction accessory protein. Among these factors OCLN is the most important as it localizes TβRI to the tight junctions to induce EMT [776]. Despite being independent of SMAD2/3, SMAD 7 appears to be required for TGF-β mediated cdc42 activation. The exact mechanism of SMAD7 in the tight junctions is not known [777].

TGF-β induced PI3K/AKT pathway:

Similar to the other non-SMAD pathways, PI3K/AKT pathway induced by TGF-β is also not dependent on SMAD2/3 activation. The first indication of TGF-β as a potential activator of the PI3K cascade came from rapid phosphorylation of Akt by TGF-β and the association of the regulatory domain of PI3K, p85 to TβRII in immunoprecipitation experiments. TGF-β can activate PI3K directly via the kinase activity of TGFRs or indirectly through TGF-β induced TGF-α expression [778]. There is a balance between the SMAD pathways and PI3K pathways induced by TGF-β and TGF-β can down regulate the activity of PI3K through SMAD dependent expression of lipid phosphatase SHIP. This may account for the transient nature of activation of Akt by TGF-β [779].

PI3K is implicated in TGF-β-mediated actin dynamics and cell migration, thus contributing to TGF-β-induced EMT. TGF-β induced PI3K causes EMT via its downstream effectors Akt and mammalian target of rapamycin (mTOR), which is a key regulator of S6 kinase (S6K) and eukaryotic initiation factor 4E-binding protein-1 (4EBP). As expected, chemical inhibition of PI3K or dominant negative forms of Akt mitigates TGF-β induced mTOR activation. Invariably, phosphorylation of S6K and 4EBP-1 induced by mTOR enhances the transcription of the target genes required for TGF-β-induced EMT [780]. Besides induction of EMT, PI3K also mediates TGF-β-dependent fibroblast proliferation and morphological transformation through the activation of tyrosine kinase C – Abl [781].

The PI3K pathway is the only non-SMAD pathway of TGF-β capable of antagonizing the SMAD dependent pathway of TGF-β. PI3K and Akt thereby protect the cells from TGF-β-induced apoptosis and growth inhibition. Akt does this by directly binding SMAD3 and inhibiting its nuclear translocation [782, 783]. Similarly, Akt can also inhibit the nuclear translocation of forkhead transcription factor (FoxO), which is a part of the activation complex consisting of SMAD3 and SMAD4 required for TGF-β induced growth inhibition [751]. In addition to Akt, mTOR and phosphor-p70 S6 kinase (SK6) are also implicated in antagonizing TGF-β mediated responses [784]. However, their precise role and associated mode of action needs further exploration.

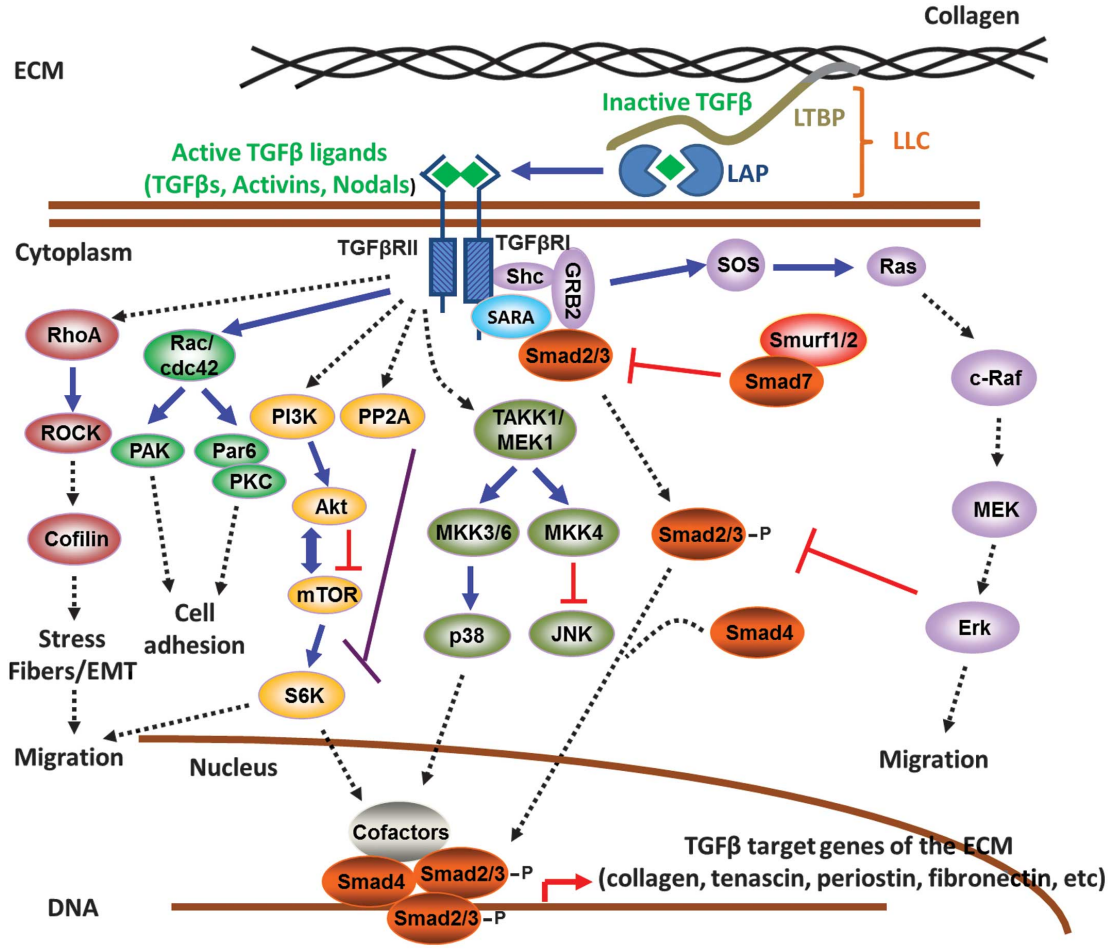


Figure 20: TGF-β signaling: Diagram depicting the canonical SMAD and non-canonical downstream effectors of TGF-β signaling. (Adapted from [785])

1.9.6 Crosstalk between TGF-β and other signaling pathways

In contrast to bFGF, the crosstalk pathways of TGF-b are mostly implicated in carcinogenesis. A series of studies carried out in breast cancer development models have shown that HER2/Neu/ErbB2 signaling which has MAPK and PI3K/Akt as its downstream effectors communes with TGF-β signaling pathways. General conclusions from these studies indicate that both positive and negative regulation exists between HER2 and TGF-β pathways. TGF-β-induced apoptosis and cell cycle arrest is antagonized by HER2 by which it promotes the pro-migratory and pro-inflammatory invasive functions of TGF-β. The synergy between TGF-β signaling and HER2/ Ras/MAPK cascade positively regulates the secretion of growth factors including TGF-β itself, which in turn leads to EMT and cell invasion [786]. In liver cancers and gliomas, TGF-β interacts with the PDGF signaling and extents its pro-oncogenic and pro-metastatic functions [787].

TGF-β and WNT signaling are intertwined throughout the life of an organism, and molecularly they interact at the ligand, cytoplasmic and nuclear levels. The ligands of TGF-β and WNT signaling are reciprocally regulated by each other to establish extracellular gradients of morphogens during embryonic development. Cytoplasmic interactions between these pathways are required for fine-tuning their respective signaling. At the nuclear level,

SMAD/β-catenin/Lef protein complexes synergistically regulate a host of shared target genes [788].

In several types of cells, SMADs modulate SHH signaling via regulating the *Gli*2 transcription, which in turn up-regulates Gli1. This modulation is of particular relevance in pancreatic cancer cells that are resistant to SHH inhibition. Therefore, blocking the function of TGF-β, which will attenuate Gli mediated cell growth, can treat SHH resistant pancreatic cancers. In the developing cerebellum, SHH stimulates the proliferation of granule cell precursors, while TGF-β signaling antagonizes the proliferative function of SHH through SMAD5 [789]. In addition, the target gene of TGF-β, TGF-β inducible early gene-1 (TIEG-1) deters Gli-mediated transcription of *N-myc*, which is an essential target of SHH, thereby inhibiting cell proliferation and promoting cell differentiation [790].

As a general characteristic of TGF-β pathway, the proper functioning of this cascade depends on its constitutive and extensive communication with other signaling cascades including NOTCH, interferon-gamma, interleukin, leading to synergistic or antagonistic effects and eventually desirable biological outcomes [788]. This is consistent with the highly context-dependent nature of TGF-β, which is adoptive and overwhelmingly complex. The molecular details of such cross talk are often conserved and underscore the biological significance of integrated signaling.

1.9.7 Functions and regulation of TGFβR signaling

Physiological role of TGF-β signaling:

The TGF-β signaling is the most conserved pathway in the animal kingdom presumably controlling life in metazoans to humans. TGF-β can influence target genes ranging from just a few in pluripotent stem cells to hundreds in differentiated cells. These effects on target genes can be positive or negative depending on the context. 'Signaling in context' is a unique property of TGF-β signaling and there are three types of contextual determinants, which dictate the transcriptional response of TGF-β in a cell [791]. The three contextual determinants are explained as follows:

- *Extracellular composition, availability and activity of the TGF-β and its receptors:* The levels and activity of different TGF-β ligands, receptors and regulators determine the overall outcome of TGF-β signaling. Extracellular factors (so called 'inputs') that affect the intensity of the TGF-β signal quantitatively alter the cellular response through finely tuned gradients [792].

- *Factors cooperating with SMAD proteins:* Factors like forkhead box H1 (FOXH1) guide SMADs to recognize activin response elements (ARE), which are involved in mesoderm differentiation. This highlights that lineage-specific transcription factors direct TGF-β to specific loci genome-wide [793]. Therefore, diverse sets of transcription factors determine the TGF-β signaling in myoblasts, pro-B cells, myeloid precursors and erythroid precursors and different transcription factors

guide SMAD proteins in differentiated cells. Finally, the levels of SMAD partners determines if the targeted gene will be activated or repressed based on the differentiation status of the cells [791].

- *Epigenetics:* DNA methylation marks, histone modifications, nucleosome positioning, non-coding RNAs and shape of the chromatin acts as determinants for TGF-β signaling. The effect of epigenetics on TGF-β is demonstrated in embryonic stem (ES) cells. Under conditions suitable for self-renewal, ES cells maintain the pluripotency-enforcing genes in an open conformation that allows transcriptional activation downstream of TGF-β signals, while the genes responsible for differentiation remain repressed and vice versa [794, 795].

Based on these context-dependent signaling, the key physiological functions of TGF-β are listed and explained below:

TGF-β in controlling gene expression:

With TGF-β classical SMAD and non-canonical pathways, it is predictable that TGF-β controls the gene expression of a variety of biological functions. In addition to modulating the pathway related genes, TGF-β also regulates expression of noncoding RNAs, such as microRNAs (miRNAs). The expression of pri-miRNA transcript is regulated both directly and indirectly by TGF-β, thus generating mature miRNAs. Among TGF-β-influenced miRNA, miRNA-200 family (miR-200a, -200b, -200c, -141, -429 and -205) is significantly dowm-regulated in the cells that are in the EMT phase in response to TGF-β. miR-200 family members are repressed indirectly by TGF-β through the induction of ZEB1 and ZEB1 (also called SMAD-interacting protein 1) [796].

TGF-β in controlling cell proliferation:

TGF-β was first identified as a potent inhibitor of cell growth in most cell types and this effect can be reversed if TGF-β is removed. TGF-β is anti-proliferative in many cell types like epithelial, endothelial, hematopoietic and glial cells and the magnitude of the anti-proliferative effect vary depending on the cell type. TGF-β exercises its anti-proliferative effect via two mechanisms. They are

- Induction of cyclin-dependent kinase inhibitors like p15, p21 and p27 [797-799]
- Elimination of proliferative drivers by repressing c-Myc and cdc25A [800, 801]

In addition to the direct inhibition, TGF-β can also inhibit cell proliferation by indirectly opposing the actions of specific growth factors like EGF and PDGF.

In contrast to anti-proliferative properties, cell proliferation can be induced in many cells types by TGF-β. TGF-β stimulates proliferation in chondrocytes, osteoblasts, mesenchymal stem cells (MSCs), fibroblasts and endothelial cells [802]. TGF-β can induce both growth and growth inhibition in the same cell type, a property that can be attributed to its 'context dependent signaling'. The molecular mechanisms underlying TGF-β driven cell proliferation

is not clear. However, in some context, growth promotion may be induced secondary to other cytokines such as PDGF [803].

TGF-β in controlling stemness and differentiation:

Differentiation control by TGF-β is a well-known physiological function of TGF-β. TGF-β modulated differentiation in a wide variety of lineages, including immune cells, blood cells and neuronal cells [804-806]. This has been exhibited in TGF-β2-deficient mice exhibiting defects in multiple organs, including lung, heart, craniofacial, limb, spinal cord, eye, inner ear and urogenital tracts [807]. Similarly, TGF-β3-deficient mice demonstrated defects in pulmonary and palate development and TGF-β1-deficient mice showed an impaired immune system [808]. In addition, double knock out of TGF-β2 and TGF-β3 caused defects in CNS [809].

Among a wide variety of TGF-β-influenced lineages, TGF-β ligands play an important role in guiding the direction and magnitude of mesenchymal differentiation. TGF-β1 is capable of inhibiting the differentiation in adipocytes and skeletal myocytes and enhances it in chondrocytes [810]. This is in accordance with TGF-β2, which also enhances differentiation towards chondrocytes [811]. Furthermore, TGF-β exerts its dual activity of promoting and repressing the expression of differentiation markers in bone matrix-depositing osteoblasts in a way depending on their level of differentiation. This notion is confirmed as inhibition of endogenous TGF-β induces osteoblast maturation. Moreover, TGF-β also controls and maintains the pluripotency of ES cells by repressing differentiation [812].

TGF-β in wound healing:

Normal wound healing is a multi-step process that involves the following steps:

1. Cell migration and inflammation
2. Proliferation of fibroblasts and ECM deposition
3. Remodeling of scar tissue.

TGF-β regulates all the steps in wound healing and promotes accelerated healing via extending its effects on multiple cells types [813].

The expression of TGF-β attains a rapid peak upon injury, progressing outward from the site of injury. Platelets store large amounts of TGF-β1 in the hemostatic plug and then release it at the site of injury. As a potent chemoattractant, TGF-β attracts monocytes and fibroblast to the sites of inflammation and repair [814, 815]. Apart from its role as chemoattractant, TGF-β also induces the expression of major ECM proteins such as fibronectin and collagens thus facilitating ECM deposition at the site of wound healing. Furthermore, TGF-β enhances ECM deposition by inhibiting the expression of MMPs and inducing the inhibitor of MMP synthesis [816]. In addition, TGF-β represses the epithelial phenotype and concomitantly enhances the expression of mesenchymal traits with increased cell motility, which contributes to wound healing. Increased TGF-β signaling at the site of injury, promotes partial or complete EMT of the epithelial cells, thereby promoting epithelial resurfacing [817].

Even though SMAD2/3 is the common downstream effector of TGF-β1, TGF-β2 and TGF-β3, they show differences in the control of wound healing. The deposition of ECM in early stages of wound healing is amplified by TGF-β1 and TGF-β2, but this does not influence the final quality of scarring as compared to the wounds that are not exposed to TGF-β [818]. The exact reasons of these differences in early stages of wound healing remain to be fully understood.

TGF-β controls the immune system:

TGF-β impacts the immune system by serving as a potent immunosuppressive cytokine. TGF-β exerts its immunosuppressive effects on both cell differentiation and cell proliferation. TGF-β2, which was originally isolated as glioblastoma-derived T-cell suppressor factor, inhibit proliferation of T-cells and is often accompanied by immunosuppression [819]. Consistent with its context-dependent signaling, TGF-β1 can also promote T-cell differentiation. TGF-β, in combination with IL-6 or IL-21, is required for the generation of IL-17 expressing pro-inflammatory T helper cells (T_H17) [820]. The presence or absence of IL-6 modulates the effects of TGF-β on T_H17 cells, which could be either regulatory or inhibitory.

Regulation of TGF-β signaling:

Given the diverse implications in various biological functions, TGF-β is a highly regulated pathway. TGF-β signaling is regulated positively and negatively through several mechanisms. Positive regulation amplifies the effects to TGF-β to actuate TGF-β-driven biological activities. Negative regulation is often through negative feedback mechanism, which occurs at the extracellular, membrane, cytoplasmic or nuclear levels. Negative regulation limits the magnitude of TGF-β signaling and terminates the biological outcome of TGF-β. Negative regulation is indispensible for formation of gradients of morphogens during the developmental processes. Besides negative feedback mechanisms, TGF-β signaling can also be negatively regulated via cross talk between other signaling pathways [821]. While there are innumerable pathways regulating TGF-β pathways, the most important positive and negative regulatory mechanisms are listed and explained below:

Positive regulation of TGF-β signaling: Positive regulation of TGF-β signaling occurs at the extracellular level through the induction of ligands. The three isoforms of TGF-β are auto- and cross-induced by each other, thus enhancing the possibility to bind to TGFRs. At the intracellular level, transcription factors that function as targets of TGF- β signaling induce the expression of TGF-β. The canonical SMAD signaling also positively modulates TGF- β through cross talk with other pathways. Under certain conditions, the RTK pathways might activate SMADs [822]. SMAD activation by RTKs is facilitated through c-Jun and ATF-2 (also called CRE-BP1). SMAD3 interacts physically with phosphorylated c-Jun and then in conjugation with SMAD4 activate the target genes [823].

Negative regulation of TGF- β pathway: Negative regulation of TGF- β signaling occurs precisely at the extracellular, receptor and cytoplasmic levels.

Extracellular antagonist: TGF-βs are secreted as latent complexes, which need the N-terminal cleavage for the release of 'mature' TGF- β. Specific antagonists tightly control the cleavage mechanisms. Two different types of antagonist have been identified. They are explained as follows:

- *Ligand binding antagonist:* These ligands bind directly to TGF-β, thus preventing the availability of TGF-β to bind to the TGFRs. These types of antagonists include noggin, chordin, cerberus and its related proteins and follistatin [824].
- *Pseudoligand-type antagonists:* These ligands bind to the receptors and acts as pseudoligands. Presence of pseudoligands renders TGFRs inaccessible to TGF-β. Mammalian lefty-1 and lefty-2 belong to this type of ligands [825].

Regulation of receptor function: Regulation at the membrane levels occurs through pseudoreceptors. BAMBI is a pesudoreceptor for serine/threonine kinase receptors, which exhibits a high structural homology to type I Ser/Thr kinases receptors but lacks the functional intracellular kinase domain. BAMBI is a common regulator of BMP, TGF-β and activin pathway. It interacts with various type I and type II receptors and abrogates the signaling cascades induced by BMPs, TGF- β and activins [826]. Similar to BAMBI, FKBP12, an FK506-binding immunophillin binds to a Leu-Pro sequence in the GS domain of TβRI and safeguards TGF-β signaling against ligand independent activation of TGFRs [827].

Negative regulation in the cytoplasm: Inhibitory SMADs (I-SMADs), ERK/MAPK pathways, transcriptional co-repressors and proteasome degradation pathways exhibit negative regulation at the cytoplasmic level. The various mechanisms involved in the cytoplasmic negative regulation are elaborated below:

- *Inhibition by I-SMADs:* I-SMADs belong to the same family as R-SMADs and Co-SMADs but they function as antagonist to the SMAD signaling pathway. In a mechanism that is similar to R-SMADs and co-SMADs, I-SMADs interact with type I receptors activated by type II receptors. The interaction of R-SMAD with the receptor is dissociated easily whereas the I-SMADs form stable complexes thus preventing the advancement of further downstream SMAD signaling. In addition, I-SMAD also complete with co-SMADs for formation of complexes with R-SMADs. The I-SMAD, SMAD7 is a potent inhibitor of TGF-β signaling and is induced by various extracellular stimuli like growth factors and mechanical stress [828].
- *ERK/MAP kinase pathway:* ERK activated by growth factors like EGF and HGF phosphorylates Ser/Thr residues in the PX(S/T) or (S/T)O motif in the linker regions of Smads. ERK induced phosphorylated R-SMADs forms complex with co-SMADs but they are not translocated to the nucleus, thereby rendering the TGF-β signaling inactive. Thus, ERK inhibits SMAD dependent TGF-β signaling. Malignant cells with activated Ras become resistant to the growth inhibitor effect of TGF- β. This might be because of the inhibition of nuclear localization of TGF-β induced SMADs, which are important for growth inhibition [829, 830].

- *Transcriptional co-repressors:* SMADs that are translocated to the nucleus and regulate transcription of target genes. The transcriptional co-activators and co-repressors influence the transcription of target genes. A homodomain protein of the TALE class, TGIF is a known co-repressor of SMADs [831]. In addition, c-Ski and its related proteins SnoN (Ski-related novel gene) also transcriptionally repress SMADs [832].
- *Proteosomal degradation of SMAD proteins:* SAMDS are tagged for ubiquitin-proteosome degradation in both ligand-dependent and ligand-independent pathways. Smurf1 interacts with SMAD1 and SMAD5 and degrades them in a ligand-independent manner, thus reducing the net levels of intracellular SMAD1 and SMAD5. SMAD2 can be degraded by the ubiquitin-proteosome system in a ligand-dependent fashion. SMAD2 activated in the presence of TGF-β translocates to the nucleus. Nuclear localized SMAD2 regulates the transcription of the genes, however, at the same time, proteasomes might degrade nuclear SMADs, resulting in irreversible termination of TGF- β signaling [833].

1.9.8 TGF-β and TGFβR signaling in the brain and the CNS

Source and expression of TGF-β and their receptors in the central nervous system:

All three mammalian isoforms of TGF-β (TGF-β1-3) are expressed in early embryonic stages in the CNS at both mRNA and protein levels. In case of healthy adult brain, TGF-β2 and TGF-β3 contribute to most of the TGF- β related immune-reactivity. TGF-β1 is virtually absent apart from its expression in meninges and choroid plexus and constitutive expression in hippocampus, cortex and thalamus [834]. Similarly, the respective TGF-β receptors (TβRI and TβRII) are largely expressed in CNS, both in the developing and adult brain. Particularly, TβRI and TβRII are significantly expressed in cortex and hippocampus [835]. The key downstream player SMAD3 is also detected in the hippocampus and cortex [836].

All cell types in the CNS can acts as a source of TGF-β and respond to TGF-β stimulation. Certain neuronal populations, predominantly large neurons (cortex, hippocampus, brain stem and spinal cord) express the receptors of TGF-β, isoforms of TGF-β and SMAD3. Throughout the CNS, TGFRs, TGF-β2 and TGF-3β are found expressed by the glial cells, astrocytes and microglia [834, 837].

The physiological functions of TGF-β in the brain:

Despite being widely expressed in CNS, the physiological role of TGF-β in the CNS is not completely dissected. However, the diverse role of TGF-β in the development of the CNS including unique functions in neuronal transmission and neuroendocrine regulations are established [838]. The physiological roles of TGF-β are explained below:

TGF-β in brain development:

During the development of the CNS, TGF-β is prominently positive in zones of neuronal differentiation, while it is less intense in zones of active proliferation. Consistent with these expression patterns, TGF-β inhibits proliferation of neural crest cells while neurogenesis actively progresses in the presence of TGF-β. Specifically, TGF-β is anti-proliferative for progenitors and the expression of neuronal markers are elevated in hippocampus and cortex. Predictably, these anti-proliferative effects are dependent on SMAD4. In addition to the role of deciding the fate of neuronal cells, TGF-β my also be involved in the differentiation of selected neuronal subtypes over other subtypes [839].

TGF-β modulates synaptic modulations:

TGF-β specifically influences synaptic transmission at central synapses, but it has a low or no influence on synaptogenesis. In addition to the central synapses, TGF-β is also essential for proper functioning of the synaptic junctions in the pre-Botzinger complex, which is a central rhythm organizer located in the brainstem [840].

Neuroendocrine functions of TGF-β:

The potential involvement of TGF-β in central reproductive regulation indicates that TGF-β might have neuroendocrine functions. TGFRs and SMAD2/3 are expressed on Gonadotropin-releasing hormone (GnRH) neurons in the preoptic area highlighting their ability to respond directly to TGF-β stimulation [841]. TGF-β1 and TGF-β3, which are secreted by the neurohypophysis, co-localize with arginine vasopressin in neurons and hypothalamus and might regulate the proliferation and secretion of anterior pituitary cells [842].

Neuroprotective mechanisms of TGF-β:

The important role played by TGF-β in the process of wound healing can be extrapolated to the neuroprotective functions of TGF-β in the brain. TGF-β contributes to neuroprotection by acting as an ant-inflammatory cytokine and by influencing scar formation. These two mechanisms are explained as follows:

- **Anti-inflammatory action:** Lesions or injury in the CNS disrupt the blood-brain barrier and thus provoke the invasion of hematogenous cells into the adjacent neural tissue. Following the invasion of hematogenous cells consisting of leukocytes, macrophages and lymphocytes, which secrete various pro-inflammatory cytokines at the site of CNS injury, resulting in inflammatory reaction and local neural degeneration. The inflammatory responses include formation of cystic cavity and activation of glial cells around the injury site. The pro-inflammatory cytokines secreted by certain T helper 1 cells such as interferon-gamma, lymphotoxin and TNF drive immunopathological processes, whereas the cytokines secreted by T helper 2 cells such as IL-10 dampen the pathological process. TGF-β guards the injury site from these collateral damages caused by the immune cells by functioning as a

potent immune suppressor via inhibition of proliferation, differentiation, activation and effector function of specific immune cells. Under certain 'context', TGF-β may also act as a pro-inflammatory agent by promoting immune evasion and serving as a chemoattractant for neutrophils [843]. Microglia, which are analogs of macrophages in the brain, are the major source of these diverse cytokines. Therefore, activation of microglia by CNS injury is a hallmark of brain pathology. Activated microglia mediate the inflammatory responses, which is complemented by the resident immune cells of the CNS that normally respond to neuronal damage and eliminate the damaged neurons by phagocytosis [838].

- **Scar Formation:** Analogous to normal wound healing, a brain injury is also followed by a cascade of cellular and molecular mechanisms resulting in a secondary injury, which is referred to as 'scar'. TGF-β is incriminated in the formation of the fibrotic scar and the glia limitans. Cells adjacent to the lesion site, express TGF-β receptors and were immune positive for fibronectin (component of ECM deposition), 3 days post injury. TGF-β also activates the astrocytes around the lesion and thus promotes astrogliosis, which is involved in glial scarring [844].

1.9.9 TGF-β in cancer and its role in tumour microenvironment

TGF-β is tied to crucial regulatory roles and when this pathway malfunctions lead to serious consequences namely tumourigenesis. Virtually all-human cell types are responsive to TGF-β. TGF-β keeps the initiation of tumours at bay, by regulating cellular proliferation, differentiation, survival, adhesion and cellular microenvironment. But genetically unstable cancer cells circumvent or alter the suppressive effects of TGF-β.

Sources of TGF-β in tumours and the tumour microenvironment:

Sustained basal levels of TGF-β are released by local sources for the maintenance of homeostasis. Under conditions of tissue injury, latent TGF-β is abundantly converted to 'mature' TGF-β and released by the platelets and various stromal components. Since, tumour mimics a 'wound that never heals', TGF-β is impeccably present in abundance in the tumour microenvironment. At the early stages of tumorigenesis, TGF-β prevents malignant progression but eventually the tumour cells use TGF-β for their own progression [845]. Many subsets of cancers have shown to express TGF-β, indicating the prominent association of TGF-β with cancer.

Sources of TGF-β within the tumour microenvironment vary from tumour to tumour. These include the cancer cells themselves or the tumour stromal cells, modulating the classical TGF-β 'context dependent signaling'. Tumours are frequently infiltrated by leukocytes, macrophages and bone marrow-derived endothelial, mesenchymal and myeloid precursor cells, which aid as sources of accumulation of TGF-β at the leading edges of invasive tumours. The accumulation of TGF-β at the invasive front of the tumour is associated with tumour progression and eventually metastasis. Furthermore, cleavage of LAP to release 'mature' TGF-β also increases the levels of TGF-β in the tumour microenvironment [846].

Deregulation of TGF-β signaling in cancer:

In order to neutralize the tumour-suppressive effects of TGF-β, cancer cells accumulate inactivating mutations in TGF-β receptors and SMAD proteins. The points of TGF-β deregulation in cancers are explained below:

Signaling Receptors: Truncated TβRII or TβRII with inactive kinases are frequently found in colon, gastric, biliary, pulmonary, ovarian, esophageal and head and neck carcinomas. The truncation or the inactivation of kinase domain of TβRII occurs due to biallelic inactivating mutations. TβRII mutations are highly associated in tumours with microsatellite instability. TβRII contains an error prone 10-base polyadenine, which remains unrepaired in microsatellite instable tumours, thus yielding mutant TβRII. Apart from this mutation, frameshift and missense mutations in the TβRI coding region are prevalent in subsets of ovarian, esophageal and head and neck cancers. In certain cancer types, high risk is associated with a hypomorphic allele (reduces gene expressed by itself), TβRI*6A. Receptor alterations can also occur at the epigenetic level, where decreased TβRI and TβRII levels are linked to the methylation of TβRI promoter. Lower than normal levels of TβRI and TβRII are observed in lung, gastric, prostate and bladder cancers [846, 847].

R-SMADS, Co-SMADS and I-SMADS: Although, R-SMADs are crucial from transducing the TGF-β signal, mutations in R-SMADs are rarely observed. A small portion of colorectal cancers harbor mutations in SMAD2 [848]. Analogous to SMAD2, loss of SMAD3 is observed in a small subset of gastric cancer and T cell lymphoblastic leukemia. SMAD2 is located in chromosome 18q21, which is vulnerable to deletion mutations. Hence, SMAD2 suffers a loss of heterozygosity in pancreatic and colon cancers [847].

In contrast to SMAD2/3, the co-SMADs are frequently mutated in cancer. Similar to SMAD2, a deletion in 18q21 affects SMAD4 by inactivating it. SMAD4 is a notable target of inactivation in pancreatic cancer and more than half of pancreatic carcinomas harbor mutations in SMAD4 [849]. In contrast to TRβII mutations, SMAD4 is mutated in more than half of sporadic colorectal cancers without microsatellite instability and in microsatellite stable esophageal cancers. Rarely, germline SMAD4 are also observed in certain cancers but generally, SMAD4 inactivation is a late stage event in tumours [847].

With regard to I-SMADs, overexpression of SMAD7 and subsequent inhibition of tumour protective effects of TGF-β is seen in endometrial carcinomas and thyroid follicular tumours [850, 851]. In immune cells, overexpression of SMAD7 predisposes the tissue to become malignant via chronic inflammation [846].

Dual role of TGF-β: TGF-β in cancer progression and regression:

The context-dependent signaling of TGF-β is responsible for its tumour suppressor as well as tumour promoter activity. This unique dual role of TGF-β has raised concerns about inhibiting TGF-β and its downstream signaling for the treatment of cancer. The various

aspects of TGF-β-induced tumour suppression and TGF-β-induced tumour progression is detailed below:

TGF-β signaling in tumour suppression:

TGF-β induced anti-proliferative effects can be impeded by either the aberrant expression of positive regulators such as cyclins and cyclin-dependent kinases (cdks) or the repression of negative regulators such as the cdk inhibitors [852]. The growth repressive effect of TGF-β is also impaired in various cancers, which downregulate the expression of c-Myc. In most tumours, TGF-β is unable to activate p15 and p21 and is incapable of downregulating c-Myc, which is most likely due to the mutations in the components of the TGF-β pathway [853].

The tumour suppressive role of TGF-β is further exemplified by functional inactivation of TGFRs, SMADS and enhanced expression of inhibitors of TGF-β signaling in human cancers and genetic mouse models of cancer development. The first target for TGF-β mediated tumour suppression is the tumour cell itself, which provides TGF-β in an autocrine manner to activate biological responses that suppress the tumour. TGF-β induced apoptosis is activated via a combination of both SMAD dependent and SMAD independent pathways and inactivation of certain SMADs impart a distinct advantage in turmorigenesis [854].

At the receptor level of TGF-β signaling, in addition to TGFRs, TGF-β also exhibits its tumour suppressive role via TβRIII. TβRIII has been considered as a tumour suppressor due to the following reasons:

1. Loss of TβRIII is often observed in human cancers [855]
2. Loss of TβRIII positively correlates with disease progression and poor patient survival and prognosis
3. Restoration of the function of TβRIII directly inhibited cancer cell migration and invasion *in vitro* and angiogenesis and metastasis *in vivo*.

Like TβRIII, TβRI and TβRII also act as tumour suppressor genes. Inactivating mutations in TβRI and TβRII are observed in human lymphoma and colon and gastric cancers, respectively [856, 857].

Tumour promoting roles of TGF-β:

Constitutively active Ras has been a long-standing mechanism in tumour progression. Indeed, TGF-β-induced Ras activity is convincingly required for tumour cell invasiveness and metastasis in squamous cell carcinoma. Although, SMAD2 can be involved in TGF-β-induced apoptosis, TGF-β activated Ras can lead to the nuclear accumulation of SMAD2, thus altering SMAD2 to transcribe genes responsible for EMT. In addition, SMAD2 induced apoptosis is also circumvented in many cancers like colorectal cancers through the upregulation of I-SMADS like SMAD6 and SMAD7. Overexpression of SMAD7 induces carcinogenesis, particularly in pancreatic cancer, where it results in the development of malignant ductal lesions with a characteristic pancreatic intraepithelial neoplasia and increased fibrosis [858, 859]. Thus, there is a possible acceleration of carcinogenesis when SMAD7 is expressed in

the early stages of cancer and blocking SMAD7 may provide new therapeutic interventions to target TGF-β signaling in cancer.

The balance between SMAD pathways and non-SMAD pathways by TGF-β coordinates the cellular responses of TGF-β to initiate epithelial-mesenchymal transdifferentiation and to determine downstream responses. For example in the case of Akt, a downstream effector of non-SMAD pathway, which interacts with SMAD3 and alters the sensitivity of various cell lines to TGF-β induced apoptosis [860]. Likewise, the cross talk between TGF-β and activation of a pro-survival pathway through NF-κB changes the sensitivity of the cancer cells to growth factors and positively correlates with cancer cell survival [861].

With TGF-β and its role in the immune system, it is evident that TGF-β is a potent regulator of T-cell, neutrophil, monocyte, macrophage, natural killer cells, cancer-associated fibroblasts and cancer-cell-autonomous signaling in the tumour microenvironment. TGF-β promotes the inherent tumour suppressive properties of T cells, neutrophils and monocytes and stimulates their migration towards the tumour. TGF-β thereby creates a tumour promoting microenvironment through recruiting specific immune cells that secrete tumour-promoting factors. Tumour cells produce TGF-β, which can suppress functional immune responses, whereas the inhibition of TGF-β in the microenvironment enhances immune recognition and destruction of tumour cells [845]. Thus, TGF-β acts as a master regulator of the cross talk between tumour and stromal cells and stromal fibroblasts induced TGF-β mediates progression of adjacent epithelium.

One of the key mechanisms of TGF-β involves the initiation of EMT, which eventually leads to metastasis. In hepatocellular carcinoma, TGF-β mediated EMT contributes to liver fibrogenesis [862]. EMT generally occurs in late stages of tumorigenesis and TGF-β promotes EMT via a combination of SMAD dependent transcription of target genes and non-SMAD effects on cell junctions. SMAD associated transcription directly or indirectly drive the activation of mesenchymal markers while non-SMAD activated RAS promotes cancer migration and dissolution of tight junction [863]. Transcriptional repressors (Snail, Slug, Lef-1, sip1) of cell adhesion, E-cadherin in particular is found activated in cells undergoing TGF-β induced EMT. Furthermore, these TGF-β induced repressors such as Snail may also promote the expression of surface markers in actively propagating 'so-called' cancer stem cells [864, 865].

1.9.10 Targeting TGF-β signaling in cancer

The dual role of TGF-β in cancer shows the inter-dependencies of cancer cells and stromal cells that display altered or no response to TGF-β. Hence, careful selection of TGF-β targeted therapy, which can be adoptive according to the context of TGF-β may affect cancer cells by indirect or microenvironment-mediated mechanisms throughout the process of carcinogenesis.

Agents targeting the TGF-β pathway for cancer therapy:

As an attractive pathway to modulate in cancer, many TGF-β pathway inhibitors have been investigated in *in vitro* pre-clinical conditions and some of which are now in clinical development. TGF-β pathway inhibition can be intervened at three levels as follows:

I. *The ligand level:* Antisense oligonucleotides can be delivered directly via intravenous injections or engineered into immune cells, which can serve as cargos and prevent TGF-β synthesis. Examples for this strategy include trabedersen (AP12009), an antisense oligonucleotide targeting TGF-β [866] and Lucanix® (belagenpumatucel-L), a TGF-β2 antisense gene-modified allogeneic cancer cell vaccine [867].

II. *The ligand receptor level:* TGF-β-neutralizing mAb and soluble receptor (also called as ligand traps) and mAB against TGFR can prevent ligand-receptor interaction. Examples for this type of intervention includes fresolimumab, a pan TGF-β antibody [868], disitertide (P144), a pediatric TGF-β1 inhibitor designed to block the interaction with its receptor and IMC-TRL (LY3022859), a mAB against TβRII [869].

III. *Intracellular level:* Classical TGF-β receptor kinase inhibitors, which can curb TGF-β signal transductions. The most potent small molecule inhibitor available for TGF-β is galunisertib (LY2157299, TβRII inhibitor). To date, LY215799 is the only TGF-β inhibitor in clinical development [870].

The list of small molecule and large molecule inhibitors of TGF-β that are under clinical development and clinical trials for oncology is summarized in the table 5

Table 5: List of small molecule and large molecule inhibitors of TGF-β signaling and their clinical trials (Adapted from [871])

Name	Targets	Trial identifier	Current status
TGFβ ligand inhibitors			
Lerdelimumab (CAT-152) Genzyme®	TGFβ2		Development stopped
Metelimumab Genzyme®	TGFβ1		Development stopped
Fresolimumab (GC1008) Genzyme®/Aventis®	TGFβ1, -β2, -β3	NCT00356460 NCT00923169 NCT01472731 NCT01112293 NCT01401062	Results in RCC, melanoma, mesothelioma and glioma; combination phase I/II in progress in breast cancer
LY2382770 Eli Lilly®	TGFβ1		In progress outside oncology
Trabedersen (AP12009) Antisens Pharma®	TGFβ2	NCT00844064 NCT00431561 NCT00761280	Results in glioma, PDAC, CRC, melanoma and glioblastoma

Lucanix (Belagenpumatucel-L) NovaRx Corporation®	TGFβ2	NCT01058785 NCT00676507	Results in glioma and NSCLC; combination phase I in progress
FANG™ Vaccine (rhGMCSF/shRNAfurin) Gradalis®	TGFβ1, -β2	NCT01061840 NCT01309230 NCT01505166 NCT01453361	In progress in melanoma, CRC and ovarian cancer
Disitertide (P144) Digna Biotech®	TGFβ1		In progress outside oncology
TGFβ receptor inhibitors			
Galunisertib (LY2157299) Eli Lilly®	TGFβRI	NCT01246986 NCT01373164 NCT01220271 NCT02178358 NCT01582269	Phase II in progress in PDAC, HCC, glioma and glioblastoma
TEW-7197 MedPacto®	TGFβRI	NCT02160106	Phase I in progress
PF-03446962 Pfizer®	ALK-1 (TGFβRI)	NCT00557856 NCT01337050 NCT01911273 NCT01486368 NCT01620970 NCT02116894	Results of phase I; phase II results pending in HCC and in progress in malignant pleural mesothelioma and refractory urothelial carcinoma; combination phase I in progress with regorafenib in CRC
IMC-TR1 (LY3022859) Eli Lilly®	TGFβRII	NCT01646203	Phase I in progress

2. Rationale and Hypothesis

- Rationale
- Hypothesis

2. Rationale and Hypothesis

2.1 Rationale

Medulloblastoma accounts for 15-20% of all pediatric brain tumors and it is the leading cause of cancer-related death in children. MB cells possess a high propensity to disseminate to the leptomeningeal spaces of the brain and spinal cord. The latest Children's Cancer Group report of 188 patients with medulloblastoma identified disseminated disease as the most powerful independent factor associated with poor survival (P=0.0006) [83]. The metastatic feature of MB cells makes aggressive, non-targeted treatment schemes necessary. Although multimodal therapy has improved the prognosis for children with MB, a substantial proportion of patients are currently incurable and MB survivors often suffer considerable treatment-related morbidities. Besides this, short-range dissemination of MB tumour cells may increase the primary tumour volume and lead to rapid onset of resistance to chemotherapy. Thus, targeted anti-dissemination / anti-metastatic therapy could be critical to combat MB by restricting tumor progression and metastatic dissemination.

The clinical reality of metastatic dissemination has been appreciated for decades. Yet, the molecular mechanisms underlying the process of dissemination remains the enigmatic aspect of MB pathogenesis. It is postulated that a diverse range of intrinsic factors (kinases, actin dynamics) and tumour microenvironment parameters [872] contribute to medulloblastoma cell motility. Hence, we hypothesize that a finer outlook of molecular events promoting medulloblastoma cell motility and brain tissue inflitration will enable the development of novel, specific therapy approaches to selectively treat metastatic medulloblastoma. Therefore, the present study addresses extrinsic and intrinsic promoters of cell dissemination in MB and explores novel molecular targeting strategies to restrict the dissemination process.

2.2 Hypothesis

Blockade of pro-migratory signaling induced by stromal / tumoral cues can prevent brain tissue infiltration in medulloblastoma.

3. Objectives and Specific Aims

- Objective
- Specific aims

3. Objectives and Specific Aims

3.1 Objective

This study was aimed to elucidate the complexities of medulloblastoma cell motility using 2D and 3D cell dissemination models. This project intended to identify tumour microenvironment parameters inducing medulloblastoma cell motility and their relationship with intrinsic factors (kinases, actin dynamics), which will facilitate the identification of potential druggable targets for an effective anti-metastatic therapy for medulloblastoma

3.2 Specific Aims

1. To investigate the relationship between tumour microenvironment parameters (growth factors and cytokines) and medulloblastoma cell dissemination.
2. To establish an *in vitro* medulloblastoma cell motility and invasion model in 2D and 3D environment.
3. To study the inter-connections between the signaling pathways promoting cell dissemination in medulloblastoma.
4. To identify and validate novel anti-dissemination / anti-metastatic therapy targets in medulloblastoma.

4. Results

- Foreword
- Manuscript 1
- Manuscript 2
- Manuscript 3

4. Results

4.1 Foreword

The results of this project are based on the 2 published manuscripts and one unpublished manuscript (prepared for submission). The different aims of this project investigated in the aforementioned manuscripts are detailed below:

Manuscript 1: The Ser/Thr kinase MAP4K4 drives c-Met-induced motility and invasiveness in a cell-based model of SHH medulloblastoma

In this manuscript we addressed the Aim1 (To investigate the relationship between tumour micro-environment parameters (growth factors and cytokines) and medulloblastoma cell dissemination) of the project. We deciphered the functional significance of the HGF-c-Met signaling pathway for MB cell dissemination. We demonstrated that HGF-induced c-Met activation enhanced the speed of migration of the individual MB cells in 2D and 3D environments. We further showed that HGF-induced motile and invasive cell behavior requires the Ser/Thr kinase MAP4K4 in MB cells. Thus, our data revealed that MAP4K4 couples growth factor signaling to actin cytoskeleton regulation, which suggests that MAP4K4 could be a promising novel anti-dissemination therapy target in MB.

Manuscript 2: Computer-assisted quantification of motile and invasive capabilities of cancer cells

In this manuscript we dealt with Aim 2 (To establish an *in vitro* medulloblastoma cell motility and invasion model in 2D and 3D environment) and Aim 1 of the project. We have developed cell-based motility platforms to quantitatively and qualitatively assess cancer cell dissemination in 2D and 3D environments in high-throughput. This platform consists of cell-based assays, imaging devices for acquisition and software solutions for the quantification of the imaging data. For each assay we developed the corresponding software tools, which either progressively quantify the area covered by cells (2D zone exclusion) or number and distance of disseminated cells (3D assays). We named the individual software packages according to their working principles and the collective program suite is referred to as automated Cell Dissemination counter (aCDc). Further, to deduce the interconnection between tumour microenvironment parameters and MB cell motility, we studied the effect of predominant growth factors / cytokines on MB cells using aCDc and found bFGF, HGF and EGF as the strongest dissemination-promoting factors in MB.

Manuscript 3 (prepared for submission): Antagonizing crosstalk between bFGF and TGF-β signaling controls tissue infiltration in medulloblastoma

Aim 3 (To study the inter-connections between the signalling pathways promoting cell dissemination in medulloblastoma) and Aim 4 (To identify and validate novel anti-dissemination / anti-metastatic therapy targets in medulloblastoma) were investigated in

this manuscript. We found that bFGF, a potent pro-migratory signaling pathway in MB, promotes mesenchymal motility via FRS2, PAK-1 and ERK1/2. We revealed that bFGF-induced mesenchymal motility in the tumor cells is countered by an inhibitory circuitry induced by TGF-β via ROCK activation and FRS2 repression. This antagonistic crosstalk between bFGF and TGF-β signaling converges at the level of FRS2, which renders it an attractive target for an anti-dissemination therapy approach in MB. We have validated FRS2 as a potential anti-metastatic therapy target in MB and have unraveled the key functions and regulation of FRS2 at the molecular level for brain tissue infiltration.

4.2 Manuscript 1

Santhana Kumar et al. SpringerPlus (2015) 4:19
DOI 10.1186/s40064-015-0784-2

SpringerPlus
a SpringerOpen Journal

RESEARCH Open Access

The Ser/Thr kinase MAP4K4 drives c-Met-induced motility and invasiveness in a cell-based model of SHH medulloblastoma

Karthiga Santhana Kumar[1†], Dimitra Tripolitsioti[1†], Min Ma[1†], Jasmin Grählert[1,2], Katja B Egli[1], Giulio Fiaschetti[1], Tarek Shalaby[1], Michael A Grotzer[1] and Martin Baumgartner[1,3*]

Abstract

Medulloblastoma (MB) comprises four molecularly and genetically distinct subgroups of embryonal brain tumors that develop in the cerebellum. MB mostly affects infants and children and is difficult to treat because of frequent dissemination of tumor cells within the leptomeningeal space. A potential promoter of cell dissemination is the c-Met proto-oncogene receptor tyrosine kinase, which is aberrantly expressed in many human tumors including MB. Database analysis showed that c-Met is highly expressed in the sonic hedgehog (SHH) subgroup and in a small subset of Group 3 and Group 4 MB tumors. Using a cell-based three-dimensional cell motility assay combined with live-cell imaging, we investigated whether the c-Met ligand HGF could drive dissemination of MB cells expressing high levels of c-Met, and determined downstream effector mechanisms of this process. We detected variable c-Met expression in different established human MB cell lines, and we found that in lines expressing high c-Met levels, HGF promoted cell dissemination and invasiveness. Specifically, HGF-induced c-Met activation enhanced the capability of the individual cells to migrate in a JNK-dependent manner. Additionally, we identified the Ser/Thr kinase MAP4K4 as a novel driver of c-Met-induced invasive cell dissemination. This increased invasive motility was due to MAP4K4 control of F-actin dynamics in structures required for migration and invasion. Thus, MAP4K4 couples growth factor signaling to actin cytoskeleton regulation in tumor cells, suggesting that MAP4K4 could present a promising novel target to be evaluated for treating growth factor-induced dissemination of MB tumors of different subgroups and of other human cancers.

Keywords: Medulloblastoma; Cancer cell dissemination; Cell motility; c-Met; MAP4K4; Actin dynamics

Background

Medulloblastoma (MB) is the most common malignant brain tumor in children and accounts for approximately 10% of all pediatric cancer deaths. MB is thought to arise from neuronal progenitor cells harboring defects in the regulation of gene expression that normally controls growth and development of the cerebellum (Roussel and Hatten 2011). MB cells can disseminate from the primary tumor in the cerebellum throughout the central nervous system and cause metastatic disease in as many as 30% of patients at diagnosis. MB comprises a diverse set of tumors (Northcott et al. 2012a) and four molecular subgroups with differential metastatic potential, named WNT (wingless), SHH (sonic hedgehog), Group 3, and Group 4 (Taylor et al. 2012), have been classified, which remain stable from primary to recurrent MB (Ramaswamy et al. 2013). Treatments that specifically target metastatic dissemination are needed to improve patient survival and reduce treatment-related morbidity.

The receptor tyrosine kinase mesenchymal epithelial transition factor (c-Met) is activated by hepatocyte growth factor/scatter factor (HGF), its only known ligand to date, which triggers phosphorylation of Tyr1230, Tyr1234, and Tyr1235 in the intracellular domain of c-Met. c-Met phosphorylation promotes the induction of various intracellular

* Correspondence: Martin.Baumgartner@kispi.uzh.ch
†Equal contributors
[1]Department of Oncology, Children's Research Center, University Children's Hospital, Zurich, Switzerland
[3]University Children's Hospital Zürich, Department of Oncology, Children's Research Center, Neuro-Oncology group, August-Forel Strasse 1, CH-8008 Zürich, Switzerland
Full list of author information is available at the end of the article

signaling pathways (Trusolino et al. 2010) to control cell proliferation, survival, and mobilization through the regulation of integrin function and cytoskeleton dynamics (Trusolino et al. 2010). Aberrant c-Met activation occurs in various human cancers in different organs, including the brain, and is associated with disease progression and metastatic dissemination (Sierra and Tsao 2011; Li et al. 2005; Joo et al. 2012).

c-Met is expressed in surgical MB specimens and MB cell lines and its expression and the expression of its ligand HGF is associated with significantly worse outcome in patients (Li et al. 2005). Along with SHH, increased expression of HGF promotes formation and growth of MB tumors in mice (Binning et al. 2008). An increased level of HGF was found sufficient to drive invasiveness of orthotopically xenografted DAOY MB cells (Li et al. 2005). No activating mutation has been reported for MB-expressed c-Met to date, whereas increased c-Met activity has been linked to proliferation, anti-apoptosis, and migration in MB (Li et al. 2005; Provencal et al. 2009; Guessous et al. 2012; Guessous et al. 2010; Kongkham et al. 2010; Onvani et al. 2012). c-Met was found to increase the expression of the transcription factor v-myc avian myelocytomatosis viral oncogene homolog (MYC) (Li et al. 2008), which is the hallmark of the most aggressive form of MB (Taylor et al. 2012). Pro-metastatic functions of c-Met are supported by the hyaluronan (HA) receptor CD44 and in particular by its transcript variant CD44v6, which supports c-Met-dependent signaling (Orian-Rousseau et al. 2002). Although CD44 expression has been associated with WNT and SHH signaling in MB, it's expression has not yet been analyzed in MB (Katoh and Katoh 2009; Asuthkar et al. 2012).

The molecular mechanisms and downstream effectors that mediate HGF-induced MB cell dissemination are incompletely understood. Herein we used cell-based *in vitro* two- and three-dimensional (2D/3D) motility assays combined with live-cell imaging and biochemical approaches to investigate and characterize potentially druggable mediators of HGF-c-Met-induced MB cell dissemination.

Results

c-Met and its co-receptor CD44 are highly expressed in a subset of MB tumors and patient derived cell lines

To determine the potential clinical relevance of c-Met in larger cohorts of MB, we compared the mRNA expression levels of c-Met in the Gilbertson, the Kool and the Delattre datasets available through the R2 platform for visualization and analysis of the microarray data. As control, we used nine cerebellum samples of patients aged between 23 and 50 years. We found that the median mRNA level of c-Met and its ligand HGF in MB tumors from these three different primary sample cohorts were clearly below that of normal human cerebellum (Figure 1A). However, a sub-population of MB tumors averaging 17.5% (Figure 1A, c-Met high) showed significantly increased c-Met expression. Moreover, the same datasets revealed high mRNA expression of the c-Met co-receptor CD44 (Orian-Rousseau et al. 2002) in all MB tumor samples. By analyzing 103 primary MB tumors of the Northcott 103 dataset (Northcott et al. 2011), Onvani *et al.* described the association of c-Met with the SHH subgroup (Onvani et al. 2012). We confirmed this finding using the 285 tumors of the MAGIC dataset (Northcott et al. 2012b) (Additional file 1: Figure S1A). An analogous but less marked association was also observed for HGF (Additional file 1: Figure S1B), but not for CD44 (Additional file 1: Figure S1C). Using quantitative real-time PCR (Figure 1B) and immunoblotting (IB) approaches (Figure 1C), we detected high c-Met, CD44, and CD44v6 expression both at the mRNA and protein levels in DAOY and UW228 cell lines, and much less (c-Met) or no (CD44/CD44v6) expression in D341 and D425 cell lines. Interestingly, three bands were detected in the anti-CD44v6 blot (Figure 1C, arrowheads), suggesting the presence of different CD44 isoforms with incorporated v6 variable region. DAOY cells are sensitive to sonic hedgehog (Gotschel et al. 2013) and considered a SHH-like MB cell line, whereas D341 is considered a group 3 cell line (Snuderl et al. 2013). We confirmed surface expression of c-Met, CD44, and CD44v6 on DAOY (Figure 1D) and UW228 cell lines (not shown) by flow cytometry. This analysis revealed that >90% of DAOY cells expressed c-Met, 100% expressed CD44, while only approximately 40% expressed the CD44v6 isoform. We therefore continued our studies by focusing specifically on c-Met and by studying what effects c-Met activation by its ligand HGF may have on cell migration and invasion and which effector pathways are needed to mediate the c-Met responses.

HGF stimulation activates JNK and MAPK/ERK pathways and promotes motility

To determine dynamics of c-Met-induced ERK and JNK activation in DAOY and UW228 cells, we stimulated the cells with HGF in a time course experiment. We found that HGF stimulation of DAOY or UW228 cells promotes rapid phosphorylation of c-Met (IB p-c-Met) within five to ten min (Figure 2A) and the concomitant activation of the downstream effector extracellular-signal-regulated kinase (ERK, IB p-ERK) (Figure 2B). c-Met and ERK phosphorylations were blocked when the cells were pretreated for 2 h with the ATP-competitive c-Met inhibitor PHA-665752 (Christensen et al. 2003) but not with the non ATP-competitive inhibitor ARQ197 (Munshi et al. 2010) (Figure 2B). However, we found that 24 h ARQ197 pretreatment was necessary to block acute, HGF-induced c-Met signaling (Figure 2C). c-Met can activate the c-Jun N-terminal kinase (JNK) (Rodrigues et al. 1997), which

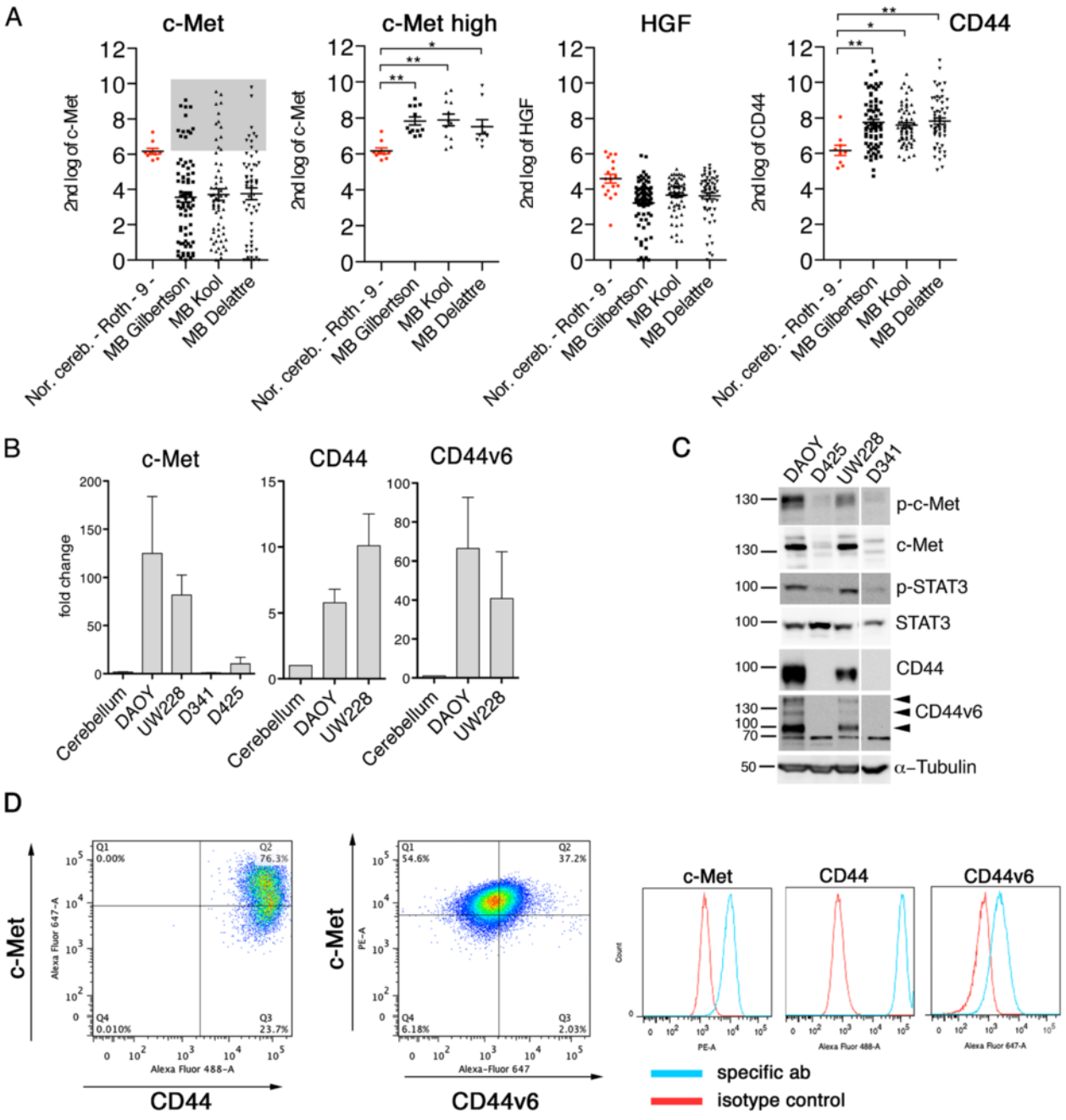


Figure 1 Expression of c-Met in medulloblastoma clinical samples and cell lines. (A) Expression analysis of c-Met, HGF and CD44 in three different MB tumor collections ($n_{total} = 195$) and in normal adult cerebellum (n = 9). **(B)** Comparative quantitative real-time PCR expression analysis of c-Met, CD44 and CD44v6 in established MB cell lines and adult cerebellum sample. **(C)** Expression and activation analysis of the c-Met pathway, CD44, and CD44v6 by immunoblotting (IB) in four different MB cell lines using the antibodies indicated to the right of the blots. **(D)** Flow cytometry analysis of DAOY cells quantifying surface expression of c-Met, CD44, and CD44v6. Dot plots compare expressions of c-Met and CD44 or c-Met and CD44v6. Histograms show relative fluorescence intensities of isotype control and specific antibody samples.

controls growth and invasion of MB cells (Zavarella et al. 2009). Consistently, we detected PHA-665752-sensitive phosphorylation of mainly the p46 isoform of JNK within five to ten minutes of HGF stimulation (Figure 2D). Interestingly, 24 h treatment with ARQ197 (Figure 2C) also caused increased JNK phosphorylation by an unknown mechanism, which was not further increase by HGF stimulation, because c-Met activity was blocked. To determine whether HGF stimulation and/or c-Met inhibition affected cell viability and/or proliferation, we performed a

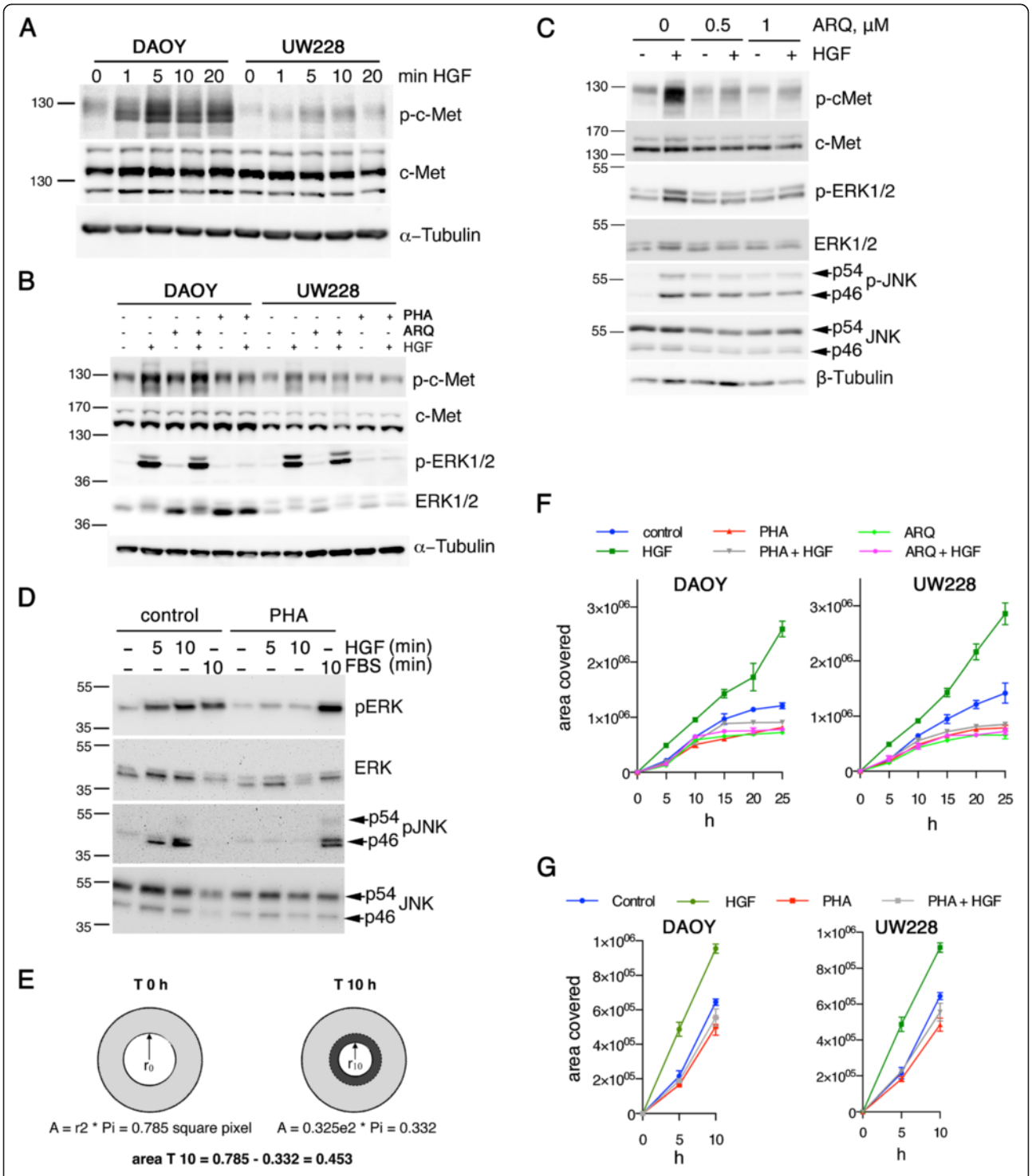


Figure 2 c-Met is activated by HGF in medulloblastoma cells and promotes wound closure. (A) IB of DAOY and UW228 lysates of cells after HGF stimulation. Antibodies used as indicated to the right of the panels. **B)** IB analysis of PHA-665752 and ARQ 197 (1 μM, 2 h pretreatment) effects on HGF-induced (50 ng/mL, 10 min) c-Met and ERK pathway activation (anti-p-c-Met and anti-pERK, respectively). **(C)** IB analysis as in B of the effect of prolonged (24 h) pretreatment with ARQ197 inhibitor on HGF-induced c-Met and ERK activation in UW228 cells. **(D)** IB analysis of HGF-induced activation of JNK and ERK in DAOY cells (20 ng/mL HGF –/+ 250 nM PHA-665752). **(E)** Schematic showing how the area covered by the cells in the Oris migration assay was determined. Light grey: cell monolayer at T_{0h}. White: gap after removal of the plug. Dark grey: area covered by cells at T_{10h}. **(F)** Quantification (means + S.D.) of Oris migration assays using DAOY or UW228 cells in HGF-treated (20 ng/mL) medium containing 0% FCS and pharmacological inhibitors (PHA-665752 and ARQ 197, both at 125 nM). **(G)** As F) but progression of gap closure shown for 0–10 h only.

tetrazolium salt WST assay on DAOY and UW228 cells treated with various combinations of HGF and PHA-665752 or ARQ197. Corresponding to c-Met expression levels (high in DAOY and UW228, low in D425), proliferation/viability was effectively reduced by the c-Met inhibitors in DAOY and UW228 cells and only moderately affected in D425 cells (Additional file 2: Figure S2). To monitor HGF-induced cell migration, we used the Oris migration assay (Gough et al. 2011) (Figure 2E) and measured the effect of HGF-c-Met signaling on the cells' capability to close a circular gap created by the insertion of a rubber stopper into the well that prevented cell attachment and growth (Figure 2F). Using time lapsed video microcopy imaging, we found that HGF treatment significantly accelerated gap closure within 24 h - both under serum-free (Figure 2F) and 10% serum (Additional file 3: Figure S3A) conditions. Importantly, time-lapse imaging showed that HGF treatment strikingly increased migration already within 5 h of incubation (Figure 2G). In DAOY cells, PHA-665752 treatment in the absence of ectopically added HGF reduced gap closure by nearly 50%, suggesting that an endogenous or a serum-derived factor activates the c-Met signaling axis and promotes pro-migratory signals (Additional file 3: Figure S3B). Overall, we showed that c-Met signaling was active in MB cells, that it was further activated by the exogenous addition of HGF and that it contributed to cell migration on 2D surfaces.

HGF promotes single cell motility and invasiveness

In assays that measure the area covered by cells such as wound healing assays or end-point Oris migration assay, it is not possible to discriminate between individual cell migration and proliferation. To determine whether HGF-induced c-Met activation indeed caused increased cell motility, we determined the speed of single cells. Towards that end, we measured the pathlength of single cells that migrated over a given time (speed) by time-lapse video microscopy. We found that HGF promoted a twofold increase in cell speed both in DAOY and UW228 cells (Figure 3A), which was blunted when c-Met was pharmacologically inhibited by either PHA-665752 or ARQ197. HGF also significantly increased single cell motility in the matrigel invasion in a c-Met-dependent manner (Figure 3B). However, the matrigel invasion assays does not permit monitoring the behavior of single cells inside a 3D matrix and measuring their speed of migration. To solve that problem, we developed a versatile micro bead invasion assay for MB cells and assessed cell dissemination from the beads into the surrounding collagen. Importantly, cells migrating inside the matrix are fully accessible for fixed- and live-cell microscopy (Figure 3C, upper). We found that HGF or epidermal growth factor (EGF) treatment promoted massive cell dissemination (Figure 3C and D). As expected, PHA-665752 prevented HGF- but not EGF-induced dissemination, confirming the specificity of this compound for the c-Met receptor tyrosine kinase. We observed that cells migrating in the collagen matrix displayed marked, F-actin rich invasive protrusions at the leading edges (Figure 3E), suggesting that local F-actin polymerization in the lamellipodia of cells is instrumental for motility. Taken together, these data demonstrate that HGF triggers dissemination of MB cells in 2D and 3D environments by accelerating motility at the single cell level. We furthermore detected enhanced local F-actin polymerization, suggesting F-actin turnover acting at the leading edge in HGF-stimulated cells as driving force.

JNK and MAP4K4 are downstream effectors of HGF-induced motility

JNK is highly expressed in the brain and controls neuronal cell migration during development (Zdrojewska and Coffey 2014) and in MB cells, HGF stimulation promoted JNK activation (Figure 2D). To test whether JNK activity was necessary for HGF-induced motility, we treated MB cells with the JNK inhibitor SP600125 (Han et al. 2001). We found that HGF-stimulated single cell motility (speed) was markedly reduced when JNK activity was blocked (Figure 4A). Interestingly, the ablation of JNK activity in the absence of HGF significantly reduced speed of single UW228 but not DAOY cells, indicating that serum-dependent motility bypasses JNK in DAOY but not in UW228 cells (Figure 4A) and suggesting different JNK pathway regulation in these closely related cell lines. We confirmed the sensitivity of HGF-induced single cell motility to JNK inhibition with the two additional JNK inhibitors JIP-1 (153–163) and AEG 3482 (Additional file 4: Figure S4). One upstream kinase of the JNK signaling pathway is the Ser/Thr kinase mitogen-activated protein kinase kinase kinase kinase 4 (MAP4K4) (Su et al. 1997). MAP4K4 mediates HGF effects on anchorage-independent growth and invasiveness (Wright et al. 2003), promotes F-actin dynamics in lamellipodia and cell motility (Baumgartner et al. 2006; Ma and Baumgartner 2014) and contributes to the progression of solid tumors in humans (Collins et al. 2006; Hao et al. 2010; Liang et al. 2008; Liu et al. 2011; Qiu et al. 2012). In human MB samples of all four subgroups, MAP4K4 is highly expressed, most significantly in the SHH and Group 4 subgroups (Additional file 1: Figure S1D). Depletion of MAP4K4 using a small interfering RNA (siRNA) approach abrogated the pro-migratory effect of HGF and also significantly reduced steady-state motility (Figure 4B). Thus, HGF-Met signaling increases speed of single migrating cells through mechanisms requiring JNK activity and MAP4K4 function, suggesting that these two kinases are essential regulators of MB cell dissemination.

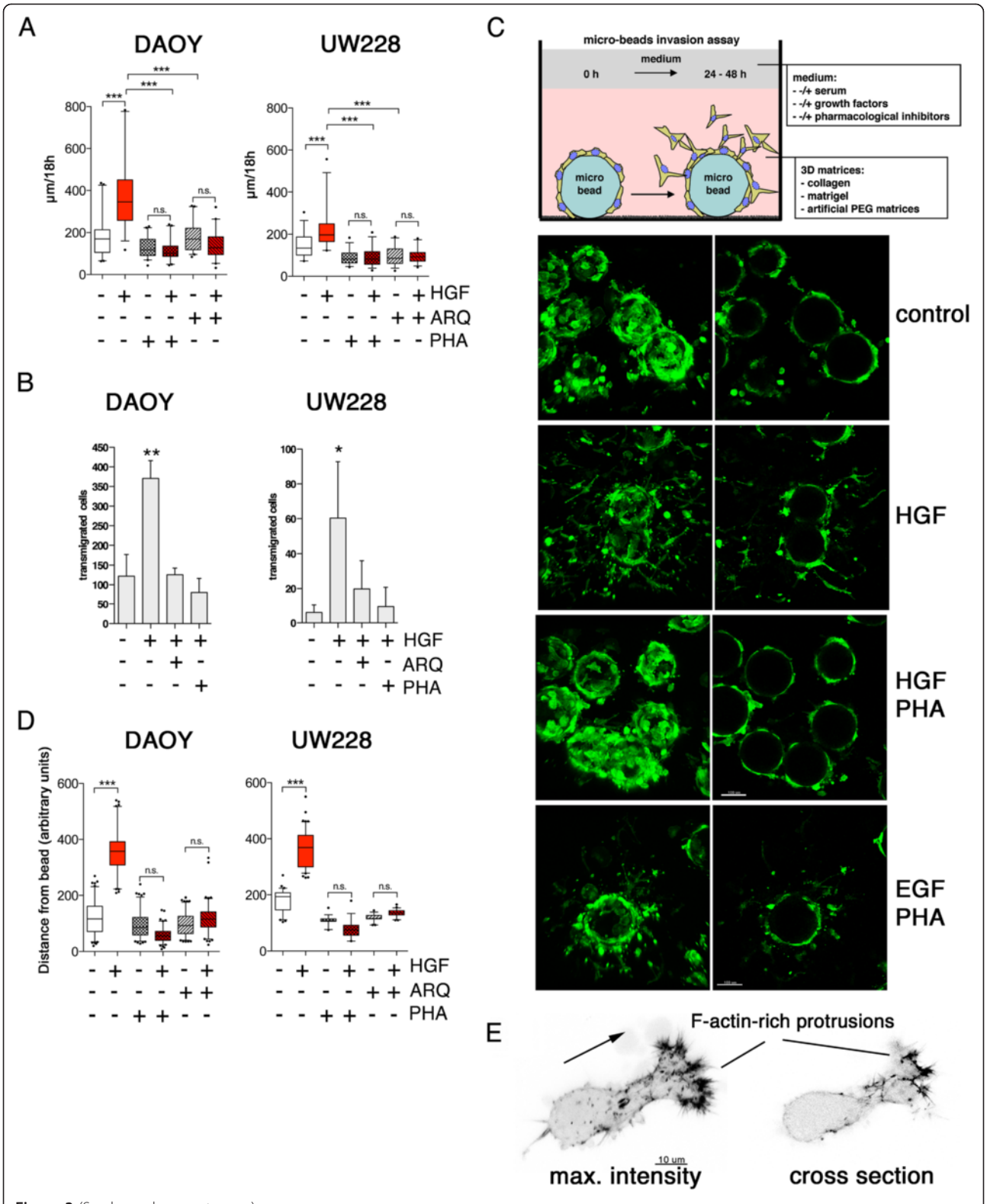


Figure 3 (See legend on next page.)

(See figure on previous page.)
Figure 3 HGF promotes invasive motility of single medulloblastoma cells. (A) Single cell motility of DAOY and UW228 cells was measured using live cell imaging (HGF: 20 ng/mL, ARQ197 and PHA-665752 250 nM). Box plots of three independent experiments are shown. **(B)** Boyden chamber invasion assay under conditions as described in (A). Mean total numbers of cells transmigrated and S.D. of representative triplicate experiment are shown. Statistical analysis: T-test, * = 0.0454, ** = 0.0038. **(C)** Upper: schematic of microbead invasion assay setup. Lower: microbeads coated with DAOY cells were embedded in collagen and cells were allowed to disseminate for 24 h. Confocal microscopy analysis of LA-EGFP fluorescence 24 h after embedding is shown (left: maximum intensity projection of Z-stacks, right: single cross-section through middle of beads). **D)** Quantification of mean and range of cell dissemination from microbeads shown in C (triplicate measurements, ten beads quantified per measurement, dot plot with SD). **E)** High-resolution confocal images of an HGF-induced (20 ng/mL) LA-EGFP expressing DAOY cell migrating in collagen. F-actin distribution is shown as inverted grey scale. Arrow: direction of migration. Note high F-actin content in invasive protrusions at leading edge of the cell.

HGF promotes cortical actin polymerization and membrane protrusion

Increased F-actin dynamics and cell motility indicated that c-Met could be active in lamellipodia to control F-actin dynamics in these structures. We used immunofluorescence (IF) microscopy to localize c-Met and p-c-Met in MB cells. Indeed, in lamellipodia of DAOY (Figure 5A, arrowheads) and UW228 (Additional file 5: Figure S5A) cells, we detected accumulations of c-Met and p-c-Met (Figure 5A, arrows). To test whether c-Met activation promoted cortical actin dynamics (Rottner and Stradal 2011), we stimulated MB cells with HGF and monitored immediate and late changes in cortical F-actin by immunofluorescence analysis (Figure 5B) and live cell imaging (movies Additional file 6: SM1, Additional file 7: SM2, Additional file 8:SM3), respectively. Interestingly, within 15 min we observed *de novo* synthesis of lamellipodial branched F-actin in the extension zone in HGF-stimulated cells (Figure 5B, magnifications), which was prevented when cells were pretreated with PHA-665752. We also observed accelerated and more prominent cortical F-actin turnover in HGF-stimulated UW228 cells (movies Additional file 6: SM1, Additional file 7: SM2, Additional file 8: SM3). To test whether MAP4K4 could promote cortical F-actin dynamics in MB cells, we expressed either enhanced green fluorescent protein (EGFP)-tagged wild-type (EGFP-MAP4K4-wt) or a kinase-defective (EGFP-MAP4K4-k/d) mutant of MAP4K4 in DAOY cells together with Lifeact fused to mCherry (LA-mCherry). We monitored F-actin dynamics by confocal live cell microscopy and quantified morphodynamic alterations of cell protrusions by kymography (Figure 5C and Additional file 5: Figure S5B). We found that F-actin polymerization dynamics in lamellipodia were significantly higher in cells expressing MAP4K4-wt and blunted in cells expressing MAP4K4-k/d. Interestingly, cells depleted of MAP4K4 by inducible short hairpin RNA expression (shRNA, see below) were also no longer able to respond to HGF stimulation with scattering (Figure 5D) and morphological alterations (contraction, measured as area covered per cell, 5E). Specifically, we observed that HGF-induced cell scattering evident in a culture of semi-confluent cells 24 h after HGF stimulation and resulting in dissociated cells with few cell-cell contacts, was abrogated by MAP4K4 depletion using shRNA. Reduced scattering may in part be due to reduced motility of single cells (Figure 5F). However, in shRNA MAP4K4-expressing cells, we also observed more cells with intact cell-cell contacts, suggesting that MAP4K4 effects on cell dissemination impact different levels of cell migration control. Taken together, our data show that MAP4K4 orchestrates HGF-induced morphodynamic processes and MB cell motility by controlling F-actin cytoskeleton dynamics and its depletion reduces the capability of MB cells to scatter in response to HGF.

MAP4K4 promotes HGF-induced single cell scattering and collagen invasion

To test whether MAP4K4 was driving HGF-induced invasive motility in collagen, we used MB cells expressing doxycycline (doxy)-inducible scrambled control shRNAs (shScr) or shRNAs targeting MAP4K4 (shMAP4K4) (Additional file 5: Figure S5C) in the micro bead invasion assay. Confocal microscopy imaging showed that HGF-promoted dissemination was markedly reduced in MAP4K4-depleted cells (Figure 6A). To quantify invasiveness of larger numbers of cells, we visualized cell nuclei (Figure 6B) and measured the distance between the bead and the individual nuclei (Figure 6C). We found that HGF-induced single cell dissemination in 3D was significantly reduced when MAP4K4 was depleted, both in 0% and 10% FCS medium. Importantly, HGF-stimulated shScr cells displayed considerably higher F-actin content at the leading edge than did MAP4K4-depleted cells (Figure 6D), indicating that MAP4K4-induced F-actin polymerization activity (Figure 5C–E) was also needed for forming invasive protrusions during cell migration in collagen. In conclusion, HGF promoted MB cell dissemination in collagen is driven by MAP4K4, probably by triggering the invasive, F-actin-rich membrane protrusions required for cells to invade and migrate (Figure 6E).

Discussion

In this study, we have investigated the functional significance of the HGF-c-Met signaling pathway for MB cell dissemination. We found that c-Met expression is upregulated in the SHH subgroup and in a subset of Group 3 and Group 4 MB tumors, as well as in some established

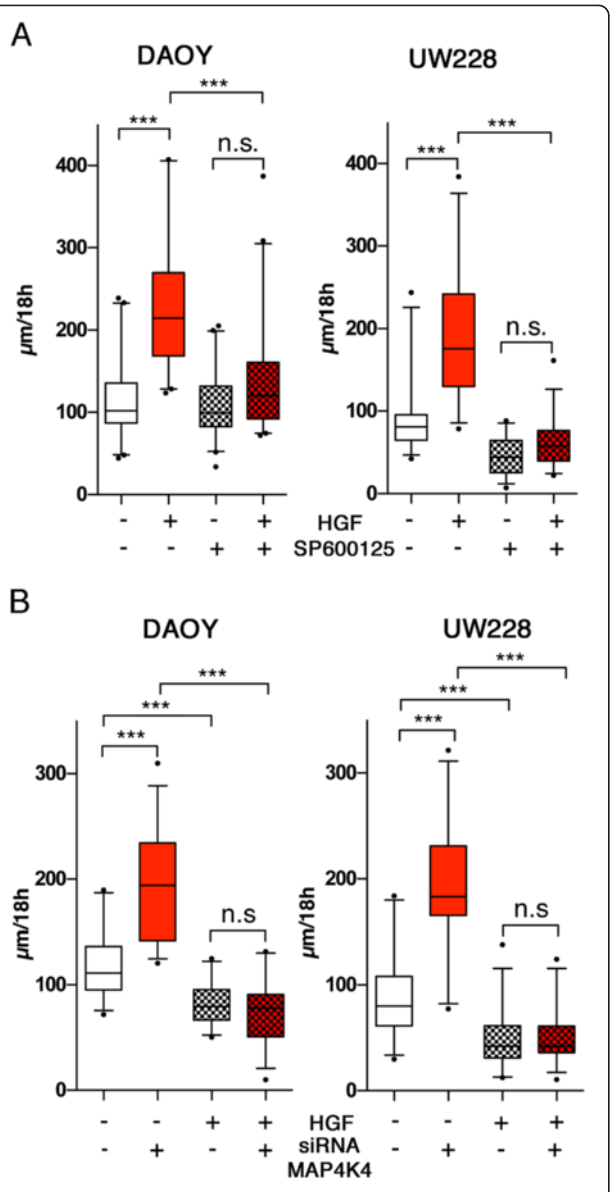


Figure 4 HGF-induced single cell motility is mediated by the Ser/Thr kinases JNK and MAP4K4. (A) Motility of single cells migrating on flat 2D surfaces in the absence or presence of HGF (20 ng/mL) and the JNK inhibitor SP665757 (10 ng/mL). Box plots of three independent experiments are shown. **(B)** Same experimental approach as in (A) but instead of using a pharmacological inhibitor, MAP4K4 was depleted from cells using validated MAP4K4-specific (+) siRNA. (–) is a negative control siRNA .

SHH MB laboratory cell lines. We demonstrated that c-Met activation by its ligand HGF promotes single cell motility of MB cells and their invasion into Matrigel and 3D collagen gels. We further showed that HGF-induced motile and invasive cell behavior requires the Ser/Thr kinase MAP4K4, which controls F-actin cytoskeleton dynamics in cellular protrusions necessary for motility and invasiveness. Thus, our studies reveal a novel, growth factor-dependent signaling circuit that promotes MB cell dissemination through MAP4K4-dependent cytoskeleton regulation, and underscore the necessity of patient stratification based on growth factor sensitivity of the tumor for rational targeting of cancer promoting signaling pathways.

Others and we have revealed a striking association of c-Met expression with SHH MB ((Onvani et al. 2012) and Additional file 1: Figure S1A) and we found that c-Met is overexpressed in approximately 18% of MB tumors compared to cerebellum controls. It is possible that c-Met could contribute to tumor progression by causing dissemination of the subset of recurrent SHH tumors reported recently (Ramaswamy et al. 2013). Importantly, c-Met function could also contribute to MB tumor cell dissemination in other subgroups by driving cell motility. However, other cellular parameters such as the capability to survive in the CSF or to colonize the new niche will be as important as well, and which could explain the discrepancy in the relative clinical outcomes between c-Met-high SHH and for example c-Met-low Group 3 tumors. Future studies examining large cohorts of patients in a subgroup-specific manner will now be required to fully appreciate the role of c-Met signaling in this context. Although the expression of the c-Met co-receptor CD44 was high in all MB tumor samples analyzed, its role in MB is unclear and further studies will also be needed here to reveal c-Met-related and un-related effects of CD44 in MB pathogenesis. Unlike CD44 expression in tumor samples, CD44 expression in MB cell lines was restricted to those expressing c-Met. Of these, only 40% co-expressed also the HGF-c-Met-interacting variant isoform CD44v6. In glioblastoma, CD44 expression conferred growth advantages and therapeutic resistance (Xu et al. 2010) and it remains to be resolved whether analogous mechanisms are also active in MB, in particular in the context of c-Met interaction with CD44v6.

Several earlier studies have implicated a role of HGF-c-Met in MB growth and dissemination and scratch wound healing assays revealed the involvement of c-Met in wound closure (Kongkham et al. 2010). However, it remained unclear whether c-Met inhibition reduced MB cell dissemination because it impaired proliferation or because it impaired cell motility. We clarified this point by providing direct evidence that HGF-c-Met function promotes the capability of MB cells to migrate, which ultimately accelerates their dissemination both in 2D and 3D environments. It can be assumed that the dual function of c-Met, stimulation of proliferation and of single cell motility is effective in other cell types or tumor cells expressing high c-Met and explains in part the effective tumorigenic activity of this receptor.

How c-Met-induced JNK promotes MB cell motility is not known; it is possible that JNK is relevant in MB cells for proper function of the microtubule skeleton during

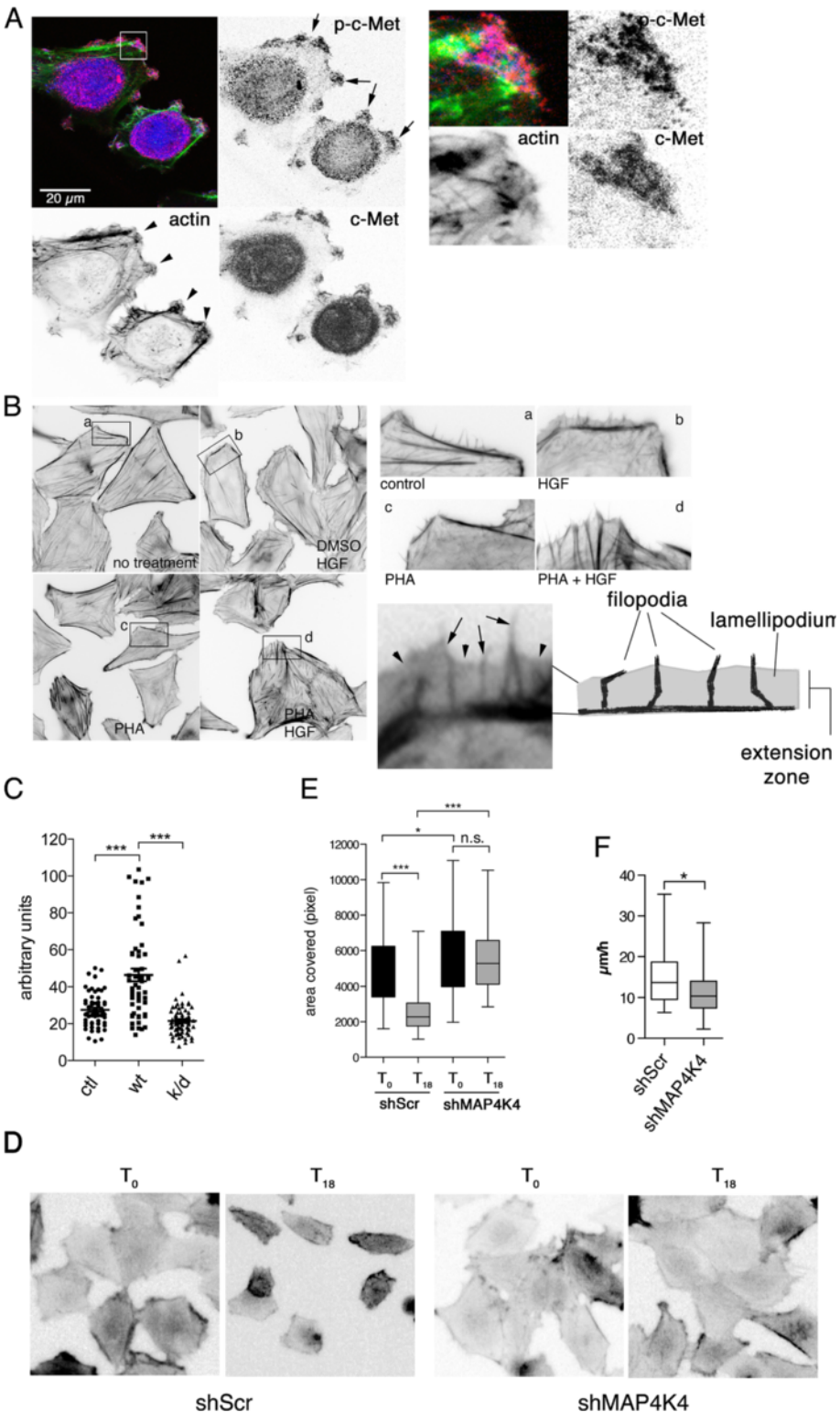


Figure 5 (See legend on next page.)

(See figure on previous page.)

Figure 5 HGF promotes cortical actin dynamics in medulloblastoma cells. (A) Immunofluorescence analysis (IFA) of c-Met and phosphorylated c-Met (p-c-Met) localization in lamellipodia of DAOY cells. Color overlay and inverted grey-scale images of p-c-Met (red), actin (green) and c-Met (blue) are shown. Magnifications are 4× of boxed area in overlay. Arrows indicate c-Met-rich lamellipodia. **(B)** IFA of Alexa-488-phalloidin-stained F-actin cytoskeletons in un-stimulated and HGF-stimulated (20 ng/mL, t = 10 min) DAOY cells, −/+ PHA-665752 (500 nM). Inverted grey-scale images of Alexa-488-phalloidin fluorescence are shown. Magnifications are 4× of boxed areas. Lower left magnification is 4× of sheet-like protrusion in b). Arrows: filopodia, arrowheads: leading edge of F-actin sheet (extension zone, see schematic). **(C)** F-actin dynamics in DAOY cells transfected with LA-mCherry and either enhanced green fluorescent protein-tagged, wild-type (wt) or kinase-defective (k/d) MAP4K4 were recorded by confocal live cell microscopy imaging. See Additional file 5: Figure S5 for still images of representative cells. Dot blots show protrusion lengths in control cells or cells expressing either EGFP-MAP4K4-wt or EGFP-MAP4K4-k/d. **(D)** Still images of time-lapse movies of DAOY-LA-EGFP-shScr or DAOY-LA-EGFP-shMAP4K4_1 cells stimulated with HGF (20 ng/mL). T_0 is 0 h and T_{18} is 18 h after HGF stimulation. Inverted grey-scale of LA-EGFP fluorescence (F-actin cytoskeleton) is shown. **(E)** Cells were treated as described in (D). Box plots of areas in pixels covered by individual cells quantified at T_0 and T_{18}. **(F)** Box plot of speeds of single sh control or shMAP4K4 cells in the presence of HGF. Statistical analysis: T-test (*: P = 0.0208).

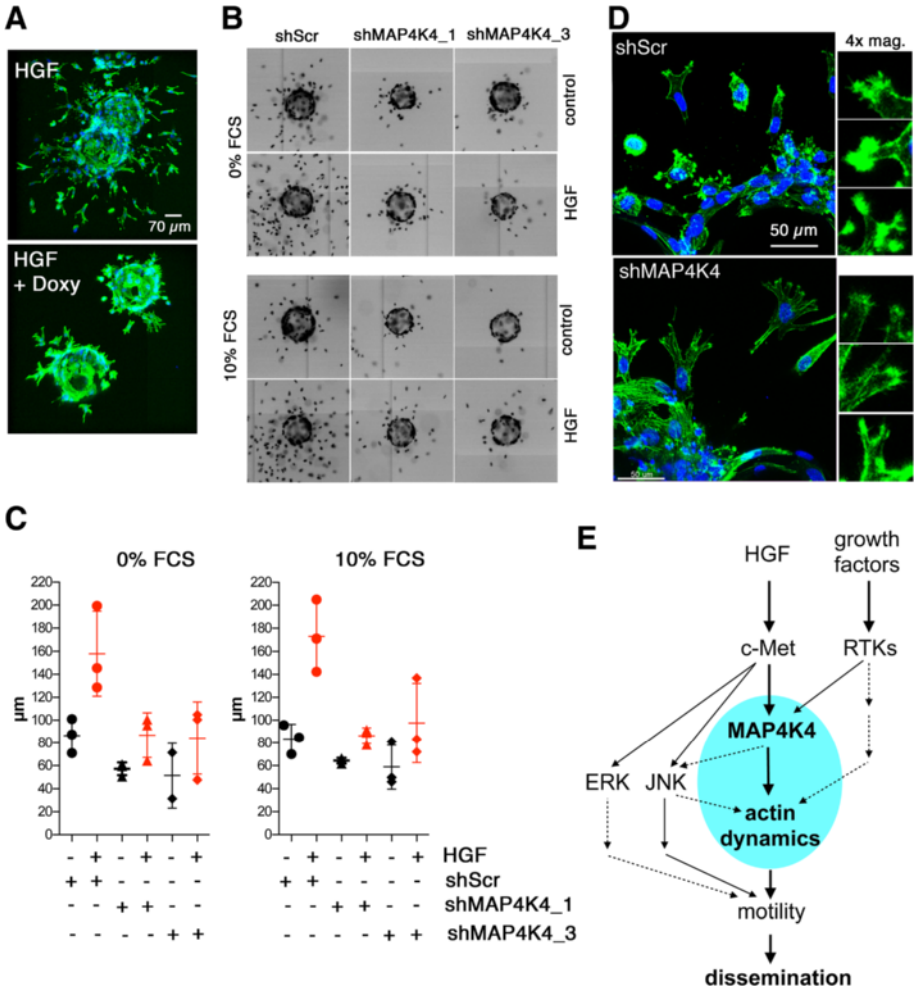


Figure 6 MAP4K4 drives HGF-induced cell dissemination in fibrillar collagen. (A) Z-stack maximum intensity projection of collagen embedded, disseminating DAOY-doxy-MAP4K4 cells. Short hairpin RNA expression was induced by doxy stimulation for 48 h before the start of the experiment (lower panel). Cells were stimulated with HGF (20 ng/mL) for 24 h. Green: F-actin, blue: DNA. **(B)** Representative montaged images of inverted grey-scale fluorescence of nuclear DNA to visualize cell dissemination. shRNA control or shMAP4K4_1/_3 expression with doxycycline and FCS and HGF treatments as indicated. **C)** Quantification of average velocities of disseminating cells with treatments as indicated. The averages of three independent experiments and S.D. are shown. **D)** High resolution confocal Z-stack of invading shScr and shMAP4K4 LA-EGFP cells. Note reduced F-actin in invasive protrusions of shMAP4K4 cells **E)** Schematic representation of signaling pathways investigated. Highlight blue is the central MAP4K4 controlled machinery that we propose to drive the dynamic remodeling of the actin cytoskeleton required for cell dissemination downstream of growth factor signaling.

motile processes through its activity toward the microtubule regulatory proteins superior cervical ganglion 10 (SCG10), doublecortin (DCX) (Zdrojewska and Coffey 2014) or microtubule-associated protein 1b (MAP1b) (Yamasaki et al. 2011). In addition to JNK, we identified MAP4K4 as a novel kinase essential for efficient dissemination of MB cells. MAP4K4 and its murine (Nck-interacting kinase), fly (misshapen) and worm (MIG15) orthologs are evolutionary conserved and control migration of both neurons (Chapman et al. 2008; Poinat et al. 2002; Shakir et al. 2006; Teuliere et al. 2011) and cancer cells (Collins et al. 2006; Wright et al. 2003; Hao et al. 2010; Liang et al. 2008; Liu et al. 2011; Qiu et al. 2012). Although its function downstream of HGF has been suggested (Wright et al. 2003), our findings are the first to demonstrate the involvement of MAP4K4 downstream of c-Met in tumor cells. Depletion of MAP4K4 reduced dissemination and the accumulation of F-actin in focal invasion structures. This finding is consistent with established functions of MAP4K4 as a regulator of cortical actin dynamics (Baumgartner et al. 2006; Poinat et al. 2002; Teuliere et al. 2011; Ma and Baumgartner 2014; Wright et al. 2003; Yan et al. 2001) and it is thus conceivable that MAP4K4 triggers and coordinates spatio-temporal actin polymerization and turnover, both of which are essential for efficient cell movement. Thus, MAP4K4 function is likely needed at the single cell level to trigger invasive cell protrusions, which in turn are necessary for motility and invasiveness of MB tumors. Although MAP4K4 is an established upstream activator of JNK (Machida et al. 2004), we could not provide convincing evidence that MAP4K4 is active in this function in MB cells as well (not shown). Hence, we concluded that while both kinases are essential for MB motility, they do probably act in parallel pathways rather than in a serial one. Considering that MAP4K4 is activated by various growth factors including HGF, PDGF (Yan et al. 2001), TNFα (Yao et al. 1999) or integrin activation (Poinat et al. 2002), we assume that several different receptor-mediated pathways trigger MAP4K4-dependent MB cell dissemination. Consequently, MAP4K4 could act as a hub to divert extracellular derived cues toward morphodynamic processes promoting motility and invasiveness (Figure 6E). Thus, we now need to further refine our understanding of upstream activators and downstream effectors of MAP4K4 in MB, because of its potential significance as a druggable anti-metastatic target for a recently synthetized novel MAP4K4 inhibitor (Crawford et al. 2014).

In summary, we have shown that the HGF-c-Met signaling pathway promotes MB cell dissemination by enabling cell dissociation, rapid movement and efficient matrix invasion of single cells. We revealed the implication of the Ser/Thr kinase MAP4K4 and its cytoskeleton modulatory functions and suggest it as a potential novel anti-metastatic target worth to investigate further. Finally, the pro-migratory functions of MAP4K4 through cytoskeleton regulation revealed herein might contribute to the metastatic progression of SHH subgroup and other MB tumors where MAP4K4 is overexpressed.

Conclusions

We have established a novel, cell-based assay to monitor cancer cell dissemination in three-dimensional matrices. We show for the first time that HGF-induced c-Met activation enhanced the speed of migration of the individual Medulloblastoma cells and show that the Ser/Thr kinase MAP4K4 is an essential mediator in this process. We conclude that MAP4K4 couples growth factor signaling to actin cytoskeleton regulation in tumor cells, suggesting that MAP4K4 could be a promising novel target to be evaluated for treating growth factor-induced dissemination of Medulloblastoma tumors of different subgroups and of other human cancers.

Methods

Ethics statement

This work was conducted according to the ethical guidelines of the University of Zürich. No donor material was used.

Expression analysis using R2 database

All data used are accessible through the open access platform R2 for visualization and analysis of the microarray data (http://r2.amc.nl). The following datasets were used: Delattre 54 MAS 5.0 – u133p2 (54 MB samples), Gilbertson 76 MAS 5.0 – u133p2 (76 pediatric MB samples, PubMed link: 22722829), Kool 62 MAS 5.0 – u133p2 (62 human MB samples, PubMed link 18769486), Northcott 103 rma_sketch – huex10t (103 primary MB samples, PubMed link 20823417) and MAGIC 285 rma-sketch – hugene11t (285 primary MB samples, PubMed link 22832581) Analysis was performed as described in (Fiaschetti et al. 2014). The nine normal cerebellum samples are from human subject aged as follows: Donor 1–25 year old male; donor 2–38 year old male; donor 3–39 year old female; donor 4–30 year old male; donor 5–35 year old male; donor 6–52 year old male; donor 7–50 year old female; donor 8–48 year old female; donor 9–53 year old female; donor 10–23 year old female.

Reagents

HGF: 0.25 μM = 20 ng/mL (Preprotech), JIP-1 (153–163) (1565, Tocris), ARQ 197 (A-1109, Active Biochemicals), PHA-665752 10 μM, AEG 3482 5 μM (Axon), (Selleck Chemicals, 10 μM). SP600125 20 μM (S5567), Doxycycline (44577) Blasticidin (15205) (Sigma-Aldrich), AEG 3482 (1291, Axon Medchem).

Cell culture

DAOY, UW228-2, D341, and D425 cells were grown as described in (Fiaschetti et al. 2014). DAOY-LA-EGFP were generated by lentiviral transduction of DAOY with cells pLenti-LA-EGFP.

Transfection

5×10^5 cells/well were seeded in 6-well plates and 24 h later transiently transfected using Jet-Pei (101–10 Polyplus), with 2.5 μg of plasmids expressing LA-mCherry (pLenti-LA-mCherry) and either MAP4K4-wt (pEGFP-C2 NIKwt) or MAP4K4-kinase dead (pEGFP-C2 NIKD152N) (Baumgartner et al. 2006).

Immunoblotting

RIPA buffer lysates were probed with the following primary and secondary antibodies: phospho-c-Met (44888, Life Technologies), c-Met (3148), phospho-STAT3 (9131), STAT3 (9132), phospho-JNK (4668), JNK (9258), phospho-ERK1/2 (9101), ERK1/2 (9102), CD44 (3578) (Cell Signaling), anti-MAP4K4 (80418, Abcam), α-tubulin (T9026, Sigma-Aldrich), and CD44v6 (MAB4073, clone VFF-18, Millipore), anti-mouse horseradish peroxidase (HRP)-linked (7076) and anti-rabbit HRP-linked (7074) (Cell Signaling). Primary antibodies were diluted 1:1000 except for α-tubulin (1:40000). Secondary antibodies were diluted 1:2000.

Immunofluorescence analysis

Cells were fixed and treated as described in (Ma and Baumgartner 2014). Primary antibodies were diluted 1:200 and incubated overnight at 4°C: α-phospho-c-Met (#44888, Life Technologies), c-Met (3148), CD44 (3578) (Cell Signaling), α-tubulin (T9026, Sigma-Aldrich), Alexa488- (A12379, Life Technologies), Cy3- (711-165-152), and Cy5-coupled (415-175-166) secondary antibodies were used (Jackson Immuno Research). Secondary antibodies and tetramethylrhodamine isothiocyanate-coupled phalloidin (Sigma-Aldrich) were diluted 1:500. Images were acquired on an Axioskop 2 mot plus fluorescence microscope (Zeiss).

Confocal live cell imaging

DAOY and UW228 cells stably expressing LA-EGFP were seeded in serum-free HEPES-buffered (25 mM) medium overnight on ibidi 8-well slides (5000 cells/well). PHA-665752 (500 nM) was added 1 h prior to and HGF (20 ng/mL) was added at the start of image acquisition in SP8 Leica confocal microscope. A 63× water immersion objective was used to acquire 60 Z-stacks of six images of EGFP fluorescence/timepoint (15 s intervals, 15 min). Average intensity projections of the stacks were assembled into QuickTime movies (10 fps, 150x speed).

Oris migration assay

The Oris™ 96-well cell migration assay kit (CMA1.101, Platypus Technologies) was used (3.5×10^4 cells seeded/well). After plug removal, cells were treated without or with HGF (20 ng/ml) and PHA-665752 or ARQ197. Cell migration was monitored for 25 h using an automated ImageXpress Micro 2 (Molecular Devices) equipped with environmental control. Images were acquired at 5 h intervals with a 10× 0.2 NA Plan Apo objective (Nikon) and Roper CoolSNAP HQ camera (Roper Scientific). Wound closure was quantified using the threshold method in the MetaXpress software (Version MX 3.1.0.93).

Matrigel invasion assay

A total of 25'000 cells were suspended in complete medium and seeded on the upper side of the Matrigel-coated membrane (BD 354480). Complete medium with or without 20 ng/ml HGF as used in the lower chamber. After 24 h, transmigrated cells were fixed with 4% PFA and stained with 0.05% crystal violet.

Single cell motility assay

Cells were seeded on 96-well glass bottom plates (In Vitro Scientific)) at 40% confluency in assay medium with or without HGF (20 ng/mL) and cell motility was acquired using the ImageXpress Micro 2 microscope. Cell speed (total path length/time) was determined by manually tracking the cells at 5 min intervals for 6–18 h using ImageJ software (National Institutes of Health, USA).

Flow cytometry

Cells were detached with Accutase (A6964, Sigma-Aldrich), fixed in 0.5% PFA for 10 min and washed in 0.5% Tween 20 (P9416, Sigma-Aldrich) and collected in flow cytometry (FC) buffer (5% FBS, 0.5% BSA, 0.1% Na-azide in PBS). 0.25×10^6 cells per sample were stained with the following primary antibodies: CD44-Alexa488 (103016, 1:50), Isotype control-Alexa488 (400625, 1:50) (BioLegend), c-Met-biotin (13–8858, 1:100), c-Met (5631, 1:100) (Cell Signaling), Isotype control-biotin (13–4301, 1:100) and CD44v6 (BMS125, 1:100) (eBioscience), and Isotype control mouse IgG1 (02–6502, Life Technologies, 1:10 – 1:50). Secondary antibodies: anti-mouse-Alexa647 (A31571, Life Technologies, 1:10000) and Streptavidin-PE (12–4317, eBioscience, 1:10000). Sequential incubations (double staining) were interrupted by three washes. Sample acquisition (10000 events) in BDFACSCanto II flow cytometer (BD Bioscience).

RNA expression analysis by qRT-PCR

Total RNA was extracted using the RNeasy Mini Kit (Qiagen, Basel, Switzerland) following the manufacturer's instructions 1 μg of total RNA was used as template for reverse transcription, which was triggered by random

hexamer primers and performed by using the High-Capacity cDNA Reverse Transcription Kit (Applied Biosystems). qRT-PCR was performed under conditions optimized for the ABI7900HT instrument, using Gene Expression Master Mix (Applied Biosystems). Probe-primer specific for the following genes (purchased from Applied Biosystems) were used: c-Met (Hs HS01565584_m1), HGF (Hs00300159_m1), CD44 (HS01075854_m1), CD44v6 (Hs01075854_m1). The relative gene expression was calculated for each gene of interest by using the ΔΔCT method, where cycle threshold (CT) values were normalized to the housekeeping gene 18S (Hs03003631_g1) (Applied Biosystems).

Microbead invasion assay

Approximately 500 Cytodex Microcarrier beads (Sigma Aldrich C3275) per 1.25×10^4 LA-EGFP-DAOY cells/ml were mixed in FACS tubes (BD Falcon **T7597-5 J**) and incubated at 37°C for 6 h, followed by incubation under rotation for 18 h. Non-adherent cells were removed. Cell-coated microbeads were resuspended in 2.5% bovine collagen I (5005-B, Advanced BioMatrix) in 96-well plate, after polymerization of collagen overlaid with fresh medium and treated with appropriate concentrations of c-Met inhibitors or HGF. After 24 h, cells were fixed with 4% PFA and stained with Hoechst. Images were acquired using the ImageXpress microscope. The distance between the microbead and the nuclei of the invaded cells was measured using ImageJ software. Velocity was calculated as the distance of displacement/time.

Generation of inducible cell lines

Inducible shRNA DAOY cell lines were generated by lentiviral transduction. Virus was produced in HEK293T using 4.5 μg of inducible pLV-H1TetO-RFP-Bsd vectors encoding either MAP4K4 shRNA (Biosettia, sh_NM_001242559 1–4) or scramble shRNA (Biosettia) along with lentivirus packaging plasmids pRev (1 μg), pMDL (3 μg), and pVSV (1.5 μg). Lentivirus-containing supernatants were added to recipient cells in the presence of 10 μg/ml of Polybrene (AL-118, Sigma,-Aldrich). At 48 h post-transduction, the culture medium was removed and stable cells were selected with 5 μg/ml blasticidin (15205, Sigma-Aldrich). Doxycycline-containing (Sigma, 44577) medium was added for 48 h for shRNA induction and protein downregulation was verified by IB and qRT-PCR.

RNA interference

The cells were transfected using either Silencer Select siRNA specific for MAP4K4 (ID: 18096) or Silencer select negative control #1 (ID: 4390843) (Ambion). Each siRNA was used at the final concentration of 5 nM in combination with Dharmafect 4 transfection reagent (Dharmacon), according to the manufacturer's instructions. MAP4K4 (ID: 18096) or Silencer select negative control #1 (ID: 4390843) (Ambion) were used. After 24, 48, and 72 h cells were harvested for both mRNA and protein extraction, to assess gene expression by qRT-PCR and protein content by immunoblotting.

Statistical analysis

Data are represented as the mean ± SD. Statistical analyses were performed using one-way analysis of variance (ANOVA) followed by Bonferroni's Multiple Comparisons test (for details please see Additional file 9: Table ST1) if not otherwise stated. P-values <0.05 were considered significant. [* < 0.05, ** < 0.01, *** < 0.001].

Additional files

Additional file 1: Figure S1. c-Met and HGF is specifically increased in the SHH subgroup of medulloblastoma. Comparison of subgroup-specific expression of **(A)** c-Met, **(B)** HGF, **(C)** CD44, and **(D)** mitogen-activated protein kinase kinase kinase kinase 4 (MAP4K4) in the MAGIC (n = 285) and Northcott (n = 103) datasets. Box plots show median, mean (+), and whiskers: 5–95 percentile.

Additional file 2: Figure S2. PHA-665752 and ARQ197 block proliferation/viability of medulloblastoma cells at low molar concentrations. DAOY and UW228 cells in medium containing 0% or 10% FCS were treated with PHA-665752 or ARQ197 as indicated. Proliferation and viability of the cells were measured using the WST assay at 0 h and after 24, 48, and 72 h.

Additional file 3: Figure S3. c-Met inhibitors block basal and HGF-induced gap closure in medium containing 10% fetal calf serum (FCS). (A) Oris migration assays using DAOY or UW228 cells in 10% FCS-containing medium treated with HGF (20 ng/mL) and c-Met inhibitors PHA-665752 and ARQ 197 (125 nM). Progression of gap closure over time expressed as area in pixels covered by cells is shown. **(B)** As A) but progression of gap closure shown for 0–10 h only.

Additional file 4: Figure S4. Pharmacological JNK inhibition blocks HGF-induced motility. Speed of single cells in the absence or presence of HGF (20 ng/mL) and the JNK inhibitors AEG 3482 (5 μM) and JIP-1 (10 μM) was acquired using live cell microscopy imaging. Path lengths of individual cells after 18 h are shown (bars = means).

Additional file 5: Figure S5. (A) IFA of c-Met and p-c-Met localization in lamellipodia of UW228 cells. Color overlay and inverted grey-scale images of p-c-Met (red), F-actin (green), and c-Met (blue) are shown. Magnifications are 4× of boxed area. Arrows indicate c-Met-rich lamellipodia. **(B)** Still images of representative cells from movies. Panels to the right of each image show kymographic analysis of protrusion along lines perpendicular to the cortical F-actin. **C)** Immunoblotting analysis of stable, doxycycline-inducible DAOY shControl (scrambled) and shMAP4K4_3 and shMAP4K4_3 cell lines after 48 h doxycycline treatment using concentrations as indicated.

Additional file 6: SM1F-actin dynamics in UW228 cells expressing Lifeact (LA)-enhanced green fluorescent protein (EGFP. 15 min recording time, 10 frames per second (fps), acceleration 150x.).

Additional file 7: SM2F-actin dynamics in HGF-stimulated (20 ng/ml, 3 h) UW228 cells expressing LA-EGFP. 15 min recording time, 10 frames per second (fps), acceleration 150x.

Additional file 8: SM3F-actin dynamics in HGF-stimulated (20 ng/ml, 3 h) UW228 cells expressing LA-EGFP treated with PHA (250 nM). 15 min recording time, 10 frames per second (fps), acceleration 150x.

Additional file 9: ST1List of statistical analyses performed.

Abbreviations
2D/3D: Two- and three-dimensional; c-Met: Mesenchymal epithelial transition factor; ERK: Extracellular-signal-regulated kinase; JNK: c-Jun N-terminal kinase; HA: Hyaluronan; HGF: Hepatocyte growth factor/scatter factor; MAP4K4: Mitogen-activated protein kinase kinase kinase kinase 4; Myc: v-myc avian myelocytomatosis viral oncogene homolog; MYCN: Avian myelocytomatosis viral oncogene neuroblastoma derived homolog; SHH: Sonic hedgehog; WNT: Wingless.

Competing interests
The authors declare that they have no competing interests.

Authors' contributions
KSM, DT, MM, JG, KE, GF and MB have carried out the experimental procedures. TS and MG have helped to draft the study. MB conceived the study and wrote the manuscript. All authors read and approved the final manuscript.

Acknowledgements
We thank John Silber for providing the UW228 cell line, Jocelyn Wright for providing MAP4K4/HGK and Olivier Pertz for providing lentiviral constructs. This study was supported by grants from the Child and Cancer Foundation to M.G., from the Swiss National Science Foundation (SNF_31004A-144090/1) to M.B., and from the University of Zürich, Forschungskredit (FK-13-039), to G.F.

Author details
[1]Department of Oncology, Children's Research Center, University Children's Hospital, Zurich, Switzerland. [2]Current address: Department of Biomedicine, University Hospital Basel, Basel, Switzerland. [3]University Children's Hospital Zürich, Department of Oncology, Children's Research Center, Neuro-Oncology group, August-Forel Strasse 1, CH-8008 Zürich, Switzerland.

Received: 22 September 2014 Accepted: 2 January 2015
Published online: 14 January 2015

References

Asuthkar S, Gondi CS, Nalla AK, Velpula KK, Gorantla B, Rao JS (2012) Urokinase-type plasminogen activator receptor (uPAR)-mediated regulation of WNT/beta-catenin signaling is enhanced in irradiated medulloblastoma cells. J Biol Chem 287(24):20576–20589, doi:10.1074/jbc.M112.348888

Baumgartner M, Sillman AL, Blackwood EM, Srivastava J, Madson N, Schilling JW, Wright JH, Barber DL (2006) The Nck-interacting kinase phosphorylates ERM proteins for formation of lamellipodium by growth factors. Proc Natl Acad Sci U S A 103(36):13391–13396

Binning MJ, Niazi T, Pedone CA, Lal B, Eberhart CG, Kim KJ, Laterra J, Fults DW (2008) Hepatocyte growth factor and sonic Hedgehog expression in cerebellar neural progenitor cells costimulate medulloblastoma initiation and growth. Cancer Res 68(19):7838–7845, doi:10.1158/0008-5472.CAN-08-1899

Chapman JO, Li H, Lundquist EA (2008) The MIG-15 NIK kinase acts cell-autonomously in neuroblast polarization and migration in C. elegans. Dev Biol 324(2):245–257, doi:S0012-1606(08)01206-2 [pii] 10.1016/j.ydbio.2008.09.014

Christensen JG, Schreck R, Burrows J, Kuruganti P, Chan E, Le P, Chen J, Wang X, Ruslim L, Blake R, Lipson KE, Ramphal J, Do S, Cui JJ, Cherrington JM, Mendel DB (2003) A selective small molecule inhibitor of c-Met kinase inhibits c-Met-dependent phenotypes in vitro and exhibits cytoreductive antitumor activity in vivo. Cancer Res 63(21):7345–7355

Collins CS, Hong J, Sapinoso L, Zhou Y, Liu Z, Micklash K, Schultz PG, Hampton GM (2006) A small interfering RNA screen for modulators of tumor cell motility identifies MAP4K4 as a promigratory kinase. Proc Natl Acad Sci U S A 103 (10):3775–3780, doi:10.1073/pnas.0600040103

Crawford TD, Ndubaku CO, Chen H, Boggs JW, Bravo BJ, Delatorre K, Giannetti AM, Gould SE, Harris SF, Magnuson SR, McNamara E, Murray LJ, Nonomiya J, Sambrone A, Schmidt S, Smyczek T, Stanley M, Vitorino P, Wang L, West K, Wu P, Ye W (2014) Discovery of selective 4-amino-pyridopyrimidine inhibitors of MAP4K4 using fragment-based lead identification and optimization. J Med Chem 57(8):3484–3493, doi:10.1021/jm500155b

Fiaschetti G, Schroeder C, Castelletti D, Arcaro A, Westermann F, Baumgartner M, Shalaby T, Grotzer MA (2014) NOTCH ligands JAG1 and JAG2 as critical pro-survival factors in childhood medulloblastoma. Acta Neuropathol Commun 2(1):39, doi:10.1186/2051-5960-2-39

Gotschel F, Berg D, Gruber W, Bender C, Eberl M, Friedel M, Sonntag J, Rungeler E, Hache H, Wierling C, Nietfeld W, Lehrach H, Frischauf A, Schwartz-Albiez R, Aberger F, Korf U (2013) Synergism between Hedgehog-GLI and EGFR signaling in Hedgehog-responsive human medulloblastoma cells induces downregulation of canonical Hedgehog-target genes and stabilized expression of GLI1. PLoS One 8(6):e65403, doi:10.1371/journal.pone.0065403

Gough W, Hulkower KI, Lynch R, McGlynn P, Uhlik M, Yan L, Lee JA (2011) A quantitative, facile, and high-throughput image-based cell migration method is a robust alternative to the scratch assay. J Biomol Screen 16(2):155–163, doi:10.1177/1087057110393340

Guessous F, Zhang Y, diPierro C, Marcinkiewicz L, Sarkaria J, Schiff D, Buchanan S, Abounader R (2010) An orally bioavailable c-Met kinase inhibitor potently inhibits brain tumor malignancy and growth. Anticancer Agents Med Chem 10(1):28–35, doi:ACA-MC-48 [pii]

Guessous F, Yang Y, Johnson E, Marcinkiewicz L, Smith M, Zhang Y, Kofman A, Schiff D, Christensen J, Abounader R (2012) Cooperation between c-Met and focal adhesion kinase family members in medulloblastoma and implications for therapy. Mol Cancer Ther 11(2):288–297, doi:10.1158/1535-7163.MCT-11-0490

Han Z, Boyle DL, Chang L, Bennett B, Karin M, Yang L, Manning AM, Firestein GS (2001) c-Jun N-terminal kinase is required for metalloproteinase expression and joint destruction in inflammatory arthritis. J Clin Invest 108(1):73–81, doi:10.1172/JCI12466

Hao JM, Chen JZ, Sui HM, Si-Ma XQ, Li GQ, Liu C, Li JL, Ding YQ, Li JM (2010) A five-gene signature as a potential predictor of metastasis and survival in colorectal cancer. J Pathol 220(4):475–489, doi:10.1002/path.2668

Joo KM, Jin J, Kim E, Ho Kim K, Kim Y, Gu Kang B, Kang YJ, Lathia JD, Cheong KH, Song PH, Kim H, Seo IHJ, Kong DS, Lee JI, Rich JN, Lee J, Nam DH (2012) MET signaling regulates glioblastoma stem cells. Cancer Res 72(15):3828–3838

Katoh Y, Katoh M (2009) Hedgehog target genes: mechanisms of carcinogenesis induced by aberrant hedgehog signaling activation. Curr Mol Med 9(7):873–886

Kongkham PN, Onvani S, Smith CA, Rutka JT (2010) Inhibition of the MET receptor tyrosine kinase as a novel therapeutic strategy in medulloblastoma. Transl Oncol 3(6):336–343

Li Y, Lal B, Kwon S, Fan X, Saldanha U, Reznik TE, Kuchner EB, Eberhart C, Laterra J, Abounader R (2005) The scatter factor/hepatocyte growth factor: c-met pathway in human embryonal central nervous system tumor malignancy. Cancer Res 65 (20):9355–9362, doi:65/20/9355 [pii] 10.1158/0008-5472.CAN-05-1946

Li Y, Guessous F, Johnson EB, Eberhart CG, Li XN, Shu Q, Fan S, Lal B, Laterra J, Schiff D, Abounader R (2008) Functional and molecular interactions between the HGF/c-Met pathway and c-Myc in large-cell medulloblastoma. Lab Invest 88(2):98–111, doi:10.1038/labinvest.3700702

Liang JJ, Wang H, Rashid A, Tan TH, Hwang RF, Hamilton SR, Abbruzzese JL, Evans DB (2008) Expression of MAP4K4 is associated with worse prognosis in patients with stage II pancreatic ductal adenocarcinoma. Clin Cancer Res 14(21):7043–7049, doi:14/21/7043 [pii] 10.1158/1078-0432.CCR-08-0381

Liu AW, Cai J, Zhao XL, Jiang TH, He TF, Fu HQ, Zhu MH, Zhang SH (2011) ShRNA-targeted MAP4K4 inhibits hepatocellular carcinoma growth. Clin Cancer Res 17(4):710–720, doi:10.1158/1078-0432.CCR-10-0331

Ma M, Baumgartner M (2014) Intracellular theileria annulata promote invasive cell motility through kinase regulation of the host actin cytoskeleton. PLoS Pathog 10(3):e1004003, doi:10.1371/journal.ppat.1004003

Machida N, Umikawa M, Takei K, Sakima N, Myagmar BE, Taira K, Uezato H, Ogawa Y, Kariya K (2004) Mitogen-activated protein kinase kinase kinase kinase 4 as a putative effector of Rap2 to activate the c-Jun N-terminal kinase. J Biol Chem 279(16):15711–15714, doi:10.1074/jbc.C300542200

Munshi N, Jeay S, Li Y, Chen CR, France DS, Ashwell MA, Hill J, Moussa MM, Leggett DS, Li CJ (2010) ARQ 197, a novel and selective inhibitor of the human c-Met receptor tyrosine kinase with antitumor activity. Mol Cancer Ther 9(6):1544–1553, doi:10.1158/1535-7163.MCT-09-1173

Northcott PA, Korshunov A, H Witt H, Hielscher T, Eberhart CG, Mack S, Bouffet E, Clifford SC, Hawkins CE, French P, Rutka JT, Pfister S, Taylor MD (2011) Medulloblastoma comprises four distinct molecular variants. J Clin Oncol 29(11):1408–1414

Northcott PA, Jones DT, Kool M, Robinson GW, Gilbertson RJ, Cho YJ, Pomeroy SL, Korshunov A, Lichter P, Taylor MD, Pfister SM (2012a) Medulloblastomics: the end of the beginning. Nat Rev Cancer 12(12):818–834, doi:10.1038/nrc3410

Northcott PA, Shih DJ, Peacock J, Garzia L, Morrissy AS, Zichner T, Stutz AM, Korshunov A, Reimand J, Schumacher SE, Beroukhim R, Ellison DW, Marshall CR, Lionel AC, Mack S, Dubuc A, Yao Y, Ramaswamy V, Luu B, Rolider A, Cavalli FM, Wang X, Remke M, Wu X, Chiu RY, Chu A, Chuah E, Corbett RD, Hoad GR, Jackman SD et al (2012b) Subgroup-specific structural variation

across 1,000 medulloblastoma genomes. Nature 488(7409):49–56, doi:10.1038/nature11327
Onvani S, Terakawa Y, Smith C, Northcott P, Taylor M, Rutka J (2012) Molecular genetic analysis of the hepatocyte growth factor/MET signaling pathway in pediatric medulloblastoma. Genes Chromosomes Cancer 51(7):675–688, doi:10.1002/gcc.21954
Orian-Rousseau V, Chen L, Sleeman JP, Herrlich P, Ponta H (2002) CD44 is required for two consecutive steps in HGF/c-Met signaling. Genes Dev 16(23):3074–3086, doi: 10.1101/gad.242602
Poinat P, De Arcangelis A, Sookhareea S, Zhu X, Hedgecock EM, Labouesse M, Georges-Labouesse E (2002) A conserved interaction between beta1 integrin/PAT-3 and Nck-interacting kinase/MIG-15 that mediates commissural axon navigation in C. elegans. Curr Biol 12(8):622–631, doi:S0960982202007649 [pii]
Provencal M, Labbe D, Veitch R, Boivin D, Rivard GE, Sartelet H, Robitaille Y, Gingras D, Beliveau R (2009) c-Met activation in medulloblastoma induces tissue factor expression and activity: effects on cell migration. Carcinogenesis 30(7):1089–1096, doi:bgp085 [pii] 10.1093/carcin/bgp085
Qiu MH, Qian YM, Zhao XL, Wang SM, Feng XJ, Chen XF, Zhang SH (2012) Expression and prognostic significance of MAP4K4 in lung adenocarcinoma. Pathol Res Pract 208(9):541–548, doi:10.1016/j.prp.2012.06.001
Ramaswamy V, Remke M, Bouffet E, Faria CC, Perreault S, Cho YJ, Shih DJ, Luu B, Dubuc AM, Northcott PA, Schuller U, Gururangan S, McLendon R, Bigner D, Fouladi M, Ligon KL, Pomeroy SL, Dunn S, Triscott J, Jabado N, Fontebasso A, Jones DT, Kool M, Karajannis MA, Gardner SL, Zagzag D, Nunes S, Pimentel J, Mora J, Lipp E et al (2013) Recurrence patterns across medulloblastoma subgroups: an integrated clinical and molecular analysis. Lancet Oncol 14(12):1200–1207, doi:10.1016/S1470-2045(13)70449-2
Rodrigues GA, Park M, Schlessinger J (1997) Activation of the JNK pathway is essential for transformation by the Met oncogene. Embo J 16(10):2634–2645, doi:10.1093/emboj/16.10.2634
Rottner K, Stradal TE (2011) Actin dynamics and turnover in cell motility. Curr Opin Cell Biol 23(5):569–578, doi:10.1016/j.ceb.2011.07.003
Roussel MF, Hatten ME (2011) Cerebellum development and medulloblastoma. Curr Top Dev Biol 94:235–282, doi:B978-0-12-380916-2.00008-5 [pii] 10.1016/B978-0-12-380916-2.00008-5
Shakir MA, Gill JS, Lundquist EA (2006) Interactions of UNC-34 Enabled with Rac GTPases and the NIK kinase MIG-15 in Caenorhabditis elegans axon pathfinding and neuronal migration. Genetics 172(2):893–913, doi:genetics.105.046359 [pii] 10.1534/genetics.105.046359
Sierra J, Tsao M-S (2011) c-MET as a potential therapeutic target and biomarker in cancer. Ther Adv Med Oncol 3(S1):S21–S35, doi:10.1177/1758834011422557
Snuderl M, Batista A, Kirkpatrick ND, Ruiz de Almodovar C, Riedemann L, Walsh EC, Anolik R, Huang Y, Martin JD, Kamoun W, Knevels E, Schmidt T, Farrar CT, Vakoc BJ, Mohan N, Chung E, Roberge S, Peterson T, Bais C, Zhelyazkova BH, Yip S, Hasselblatt M, Rossig C, Niemeyer E, Ferrara N, Klagsbrun M, Duda DG, Fukumura D, Xu L, Carmeliet P et al (2013) Targeting placental growth factor/neuropilin 1 pathway inhibits growth and spread of medulloblastoma. Cell 152(5):1065–1076, doi:10.1016/j.cell.2013.01.036
Su YC, Han J, Xu S, Cobb M, Skolnik EY (1997) NIK is a new Ste20-related kinase that binds NCK and MEKK1 and activates the SAPK/JNK cascade via a conserved regulatory domain. Embo J 16(6):1279–1290
Taylor MD, Northcott PA, Korshunov A, Remke M, Cho YJ, Clifford SC, Eberhart CG, Parsons DW, Rutkowski S, Gajjar A, Ellison DW, Lichter P, Gilbertson RJ, Pomeroy SL, Kool M, Pfister SM (2012) Molecular subgroups of medulloblastoma: the current consensus. Acta Neuropathol 123(4):465–472, doi:10.1007/s00401-011-0922-z
Teuliere J, Gally C, Garriga G, Labouesse M, Georges-Labouesse E (2011) MIG-15 and ERM-1 promote growth cone directional migration in parallel to UNC-116 and WVE-1. Development 138(20):4475–4485, doi:10.1242/dev.061952
Trusolino L, Bertotti A, Comoglio PM (2010) MET signalling: principles and functions in development, organ regeneration and cancer. Nat Rev Mol Cell Biol 11(12):834–848, doi:nrm3012 [pii] 10.1038/nrm3012
Wright JH, Wang X, Manning G, LaMere BJ, Le P, Zhu S, Khatry D, Flanagan PM, Buckley SD, Whyte DB, Howlett AR, Bischoff JR, Lipson KE, Jallal B (2003) The STE20 kinase HGK is broadly expressed in human tumor cells and can modulate cellular transformation, invasion, and adhesion. Mol Cell Biol 23(6):2068–2082
Xu Y, Stamenkovic I, Yu Q (2010) CD44 attenuates activation of the hippo signaling pathway and is a prime therapeutic target for glioblastoma. Cancer Res 70(6):2455–2464, doi:10.1158/0008-5472.CAN-09-2505
Yamasaki T, Kawasaki H, Arakawa S, Shimizu K, Shimizu S, Reiner O, Okano H, Nishina S, Azuma N, Penninger JM, Katada T, Nishina H (2011) Stress-activated protein kinase MKK7 regulates axon elongation in the developing cerebral cortex. J Neurosci 31(46):16872–16883, doi:10.1523/JNEUROSCI. 1111-11.2011
Yan W, Barber DL, Nehrke K (2001) The Nck-induced kinase NIK phosphorylates and activates the Na-H exchanger NHE1 independently of MEKK1. J Biol Chem 276:31349–31356
Yao Z, Zhou G, Wang XS, Brown A, Diener K, Gan H, Tan TH (1999) A novel human STE20-related protein kinase, HGK, that specifically activates the c-Jun N-terminal kinase signaling pathway. J Biol Chem 274(4):2118–2125
Zavarella S, Nakada M, Belverud S, Coniglio SJ, Chan A, Mittler MA, Schneider SJ, Symons M (2009) Role of Rac1-regulated signaling in medulloblastoma invasion. Laboratory investigation. J Neurosurg Pediatr 4(2):97–104, doi:10.3171/2009.4.PEDS08322 [pii] 10.3171/2009.4.PEDS08322
Zdrojewska J, Coffey ET (2014) The impact of JNK on neuronal migration. Adv Exp Med Biol 800:37–57, doi:10.1007/978-94-007-7687-6_3

Manuscript 1: Supplementary figures

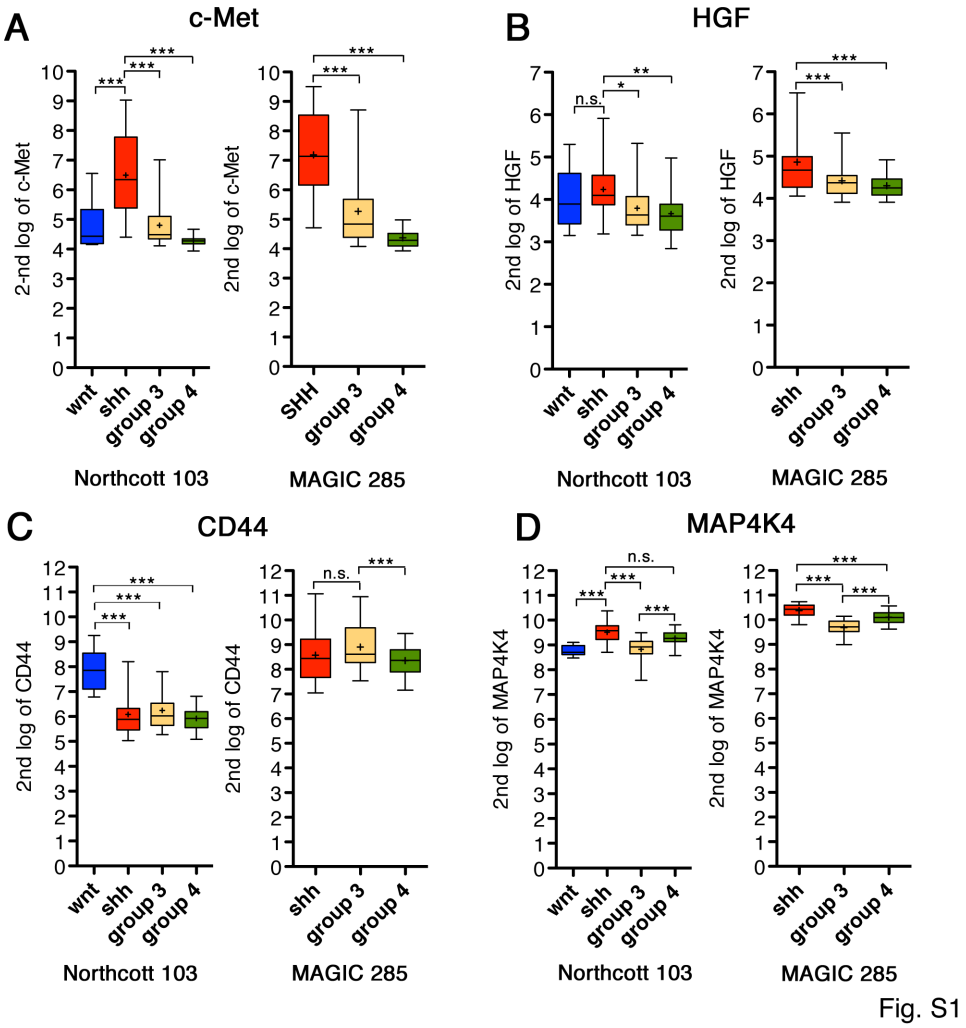


Fig. S1

Figure S1: c-Met and HGF is specifically increased in the SHH subgroup of medulloblastoma. Comparison of subgroup-specific expression of **(A)** c-Met, **(B)** HGF, **(C)** CD44, and **(D)** mitogen-activated protein kinase kinase kinase kinase 4 (MAP4K4) in the MAGIC (n = 285) and Northcott (n = 103) datasets. Box plots show median, mean (+), and whiskers: 5–95 percentile.

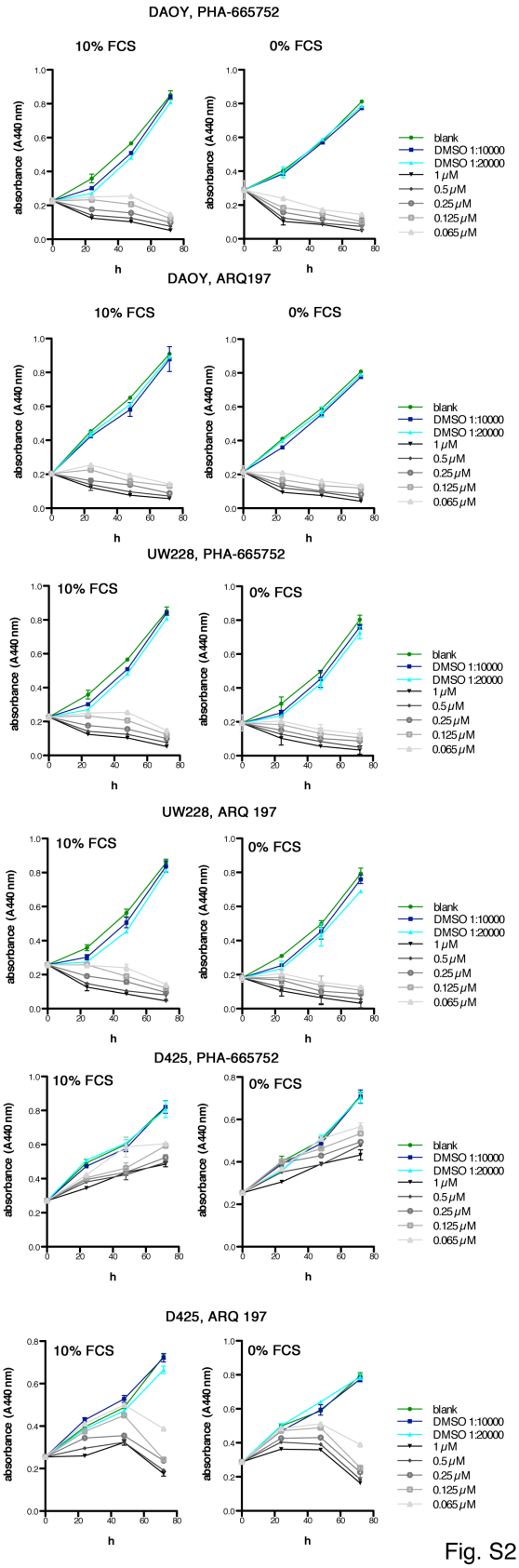


Fig. S2

Figure S2: PHA-665752 and ARQ197 block proliferation/viability of medulloblastoma cells at low molar concentrations. DAOY and UW228 cells in medium containing 0% or 10% FCS were treated with PHA-665752 or ARQ197 as indicated. Proliferation and viability of the cells were measured using the WST assay at 0 h and after 24, 48, and 72 h.

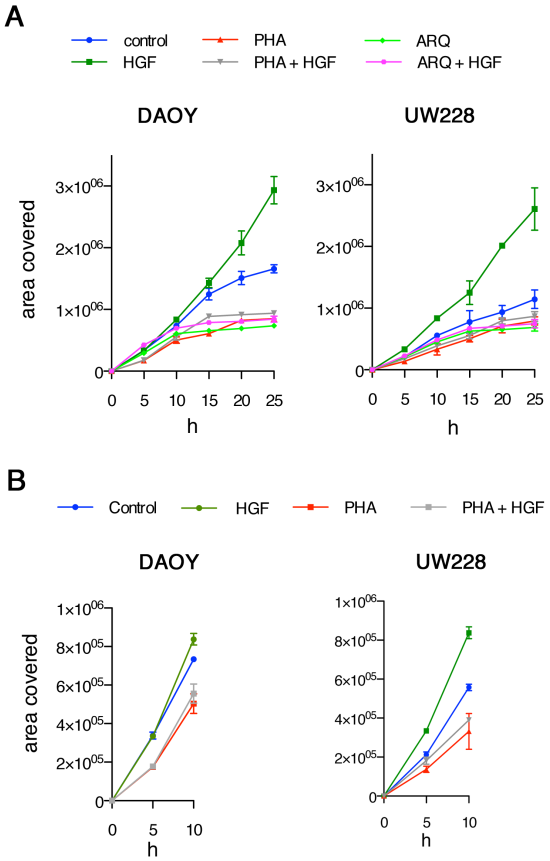


Figure S3: c-Met inhibitors block basal and HGF-induced gap closure in medium containing 10% fetal calf serum (FCS). (A) Oris migration assays using DAOY or UW228 cells in 10% FCS-containing medium treated with HGF (20 ng/mL) and c-Met inhibitors PHA-665752 and ARQ 197 (125 nM). Progression of gap closure over time expressed as area in pixels covered by cells is shown. **(B)** As A) but progression of gap closure shown for 0–10 h only.

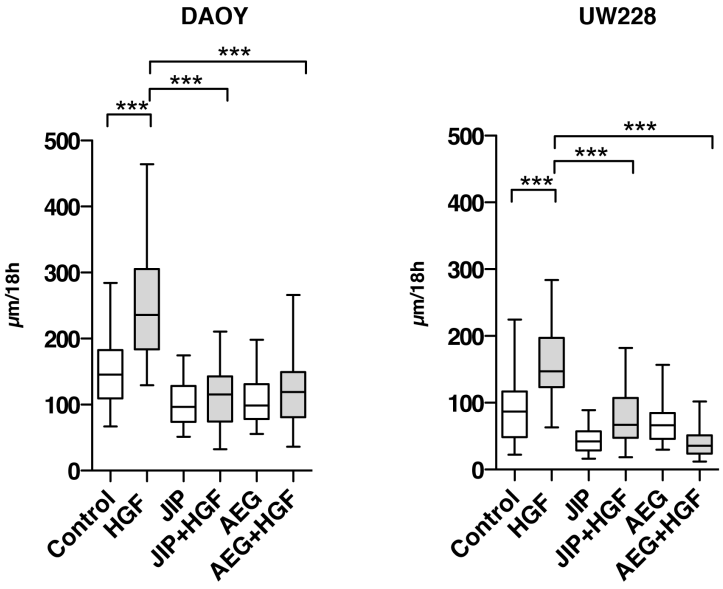


Fig. S4

Figure S4: Pharmacological JNK inhibition blocks HGF-induced motility. Speed of single cells in the absence or presence of HGF (20 ng/mL) and the JNK inhibitors AEG 3482 (5 µM) and JIP-1 (10 µM) was acquired using live cell microscopy imaging. Path lengths of individual cells after 18 h are shown (bars = means).

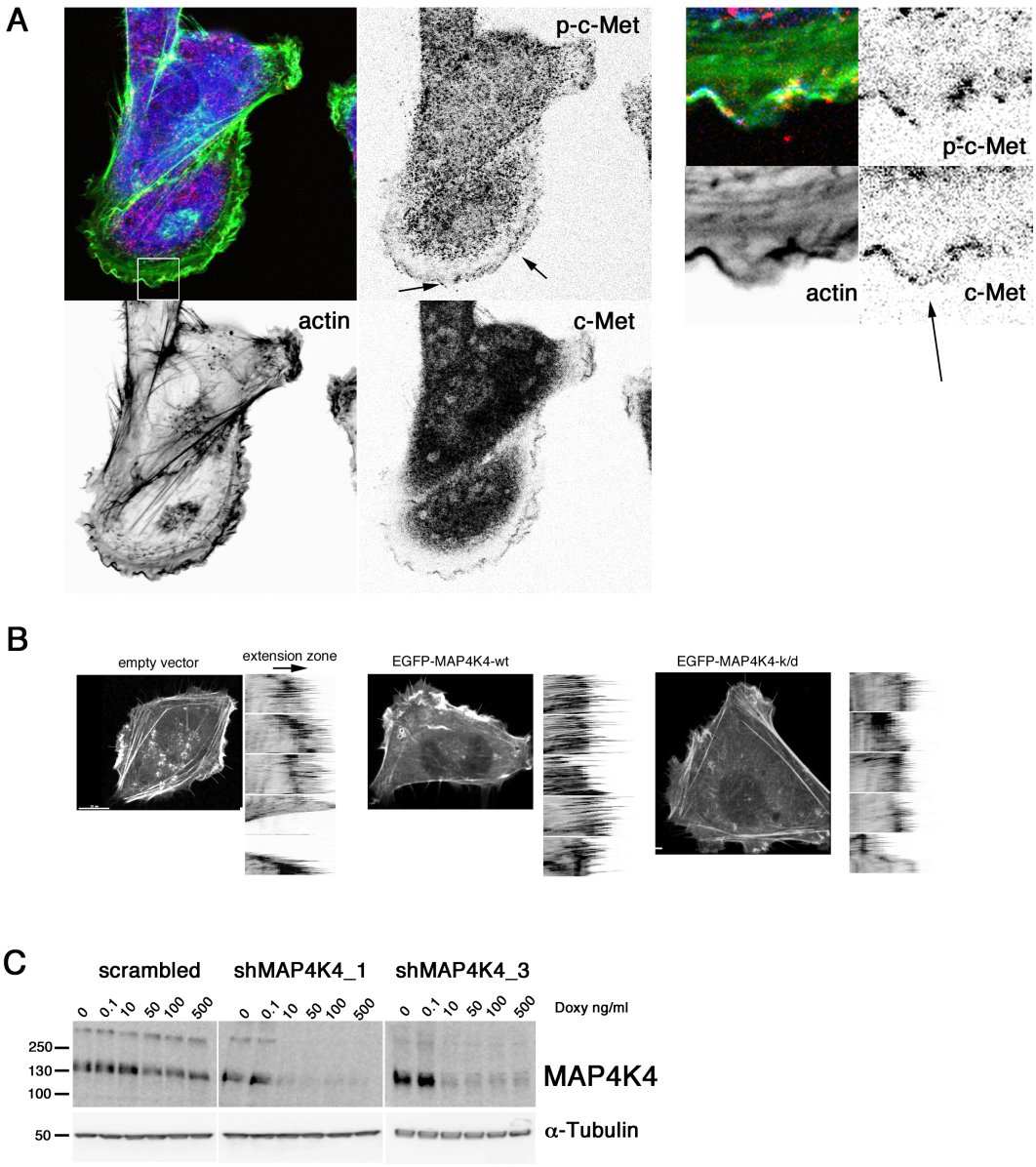


Fig. S5

Figure S5: (A) IFA of c-Met and p-c-Met localization in lamellipodia of UW228 cells. Color overlay and inverted grey-scale images of p-c-Met (red), F-actin (green), and c-Met (blue) are shown. Magnifications are 4× of boxed area. Arrows indicate c-Met-rich lamellipodia. **(B)** Still images of representative cells from movies. Panels to the right of each image show kymographic analysis of protrusion along lines perpendicular to the cortical F-actin. **C)** Immunoblotting analysis of stable, doxycycline-inducible DAOY shControl (scrambled) and shMAP4K4_3 and shMAP4K4_3 cell lines after 48 h doxycycline treatment using concentrations as indicated.

4.3 Manuscript 2

SCIENTIFIC REPORTS

OPEN

Computer-assisted quantification of motile and invasive capabilities of cancer cells

Received: 22 June 2015
Accepted: 23 September 2015
Published: 21 October 2015

Karthiga Santhana Kumar[1], Max Pillong[2,*], Jens Kunze[2,*], Isabel Burghardt[3], Michael Weller[3], Michael A. Grotzer[1,4], Gisbert Schneider[2] & Martin Baumgartner[1]

High-throughput analysis of cancer cell dissemination and its control by extrinsic and intrinsic cellular factors is hampered by the lack of adequate and efficient analytical tools for quantifying cell motility. Oncology research would greatly benefit from such a methodology that allows to rapidly determine the motile behaviour of cancer cells under different environmental conditions, including inside three-dimensional matrices. We combined automated microscopy imaging of two- and three-dimensional cell cultures with computational image analysis into a single assay platform for studying cell dissemination in high-throughput. We have validated this new approach for medulloblastoma, a metastatic paediatric brain tumour, in combination with the activation of growth factor signalling pathways with established pro-migratory functions. The platform enabled the detection of primary tumour and patient-derived xenograft cell sensitivity to growth factor-dependent motility and dissemination and identified tumour subgroup-specific responses to selected growth factors of excellent diagnostic value.

Cell migration is fundamental for numerous cellular physiological processes and the de-regulation of its homeostatic control is causative for human diseases ranging from autoimmunity and inflammation to cancer metastasis[1–3]. Cell migration is controlled by the integration of mechanical and chemical cues and their impact on the executing machinery, the cellular cytoskeleton[4–6], which defines cellular morphology and morphodynamics by a broad range of processes[7–9]. Hence, aberrant induction and maintenance of a migratory phenotype could be caused by a plethora of molecular processes coupled to cellular morphodynamics. Deeper insights into these processes and the systematic study of the underlying mechanisms require innovative, high-throughput tools that enable multidimensional visualization and quantification of cell motile behaviour in space and time.

According to the World Cancer Report 2014 of the World Health Organization metastatic dissemination of tumour cells is the leading cause of death in cancer patients, and understanding of the causative events of cancer metastasis will be essential for developing effective targeting strategies[3,10]. The identification of the relevant cellular processes remains a formidable challenge because of the large number of potential targets to be explored and the difficulties to reproducibly track altered cell motility. Cell migration is a graded process, with small alterations caused by subtle changes in the cellular motility machinery. Many cell-based assays have been developed to monitor the behaviour of cells on two-dimensional (2D) surfaces or inside three-dimensional (3D) matrices[11–15]. Several assays tackled high-throughput quantification of cell motility in 2D[15–17]. Assays to automatically determine the dissemination range of

[1]Department of Oncology, Children's Research Center, University Children's Hospital Zürich, August-Forel Strasse 1, CH-8008 Zürich, Switzerland. [2]Department of Chemistry and Applied Biosciences, ETH Zürich, Vladimir-Prelog-Weg 4, CH-8093 Zürich, Switzerland. [3]Department of Neurology, University Hospital Zürich and University of Zürich, Frauenklinikstrasse 26, CH-8091 Zürich, Switzerland. [4]Department of Oncology, University Children's Hospital Zürich, Steinwiesstrasse 75, CH-8032 Zürich, Switzerland. *These authors contributed equally to this work. Correspondence and requests for materials should be addressed to M.B. (email: Martin.Baumgartner@kispi.uzh.ch)

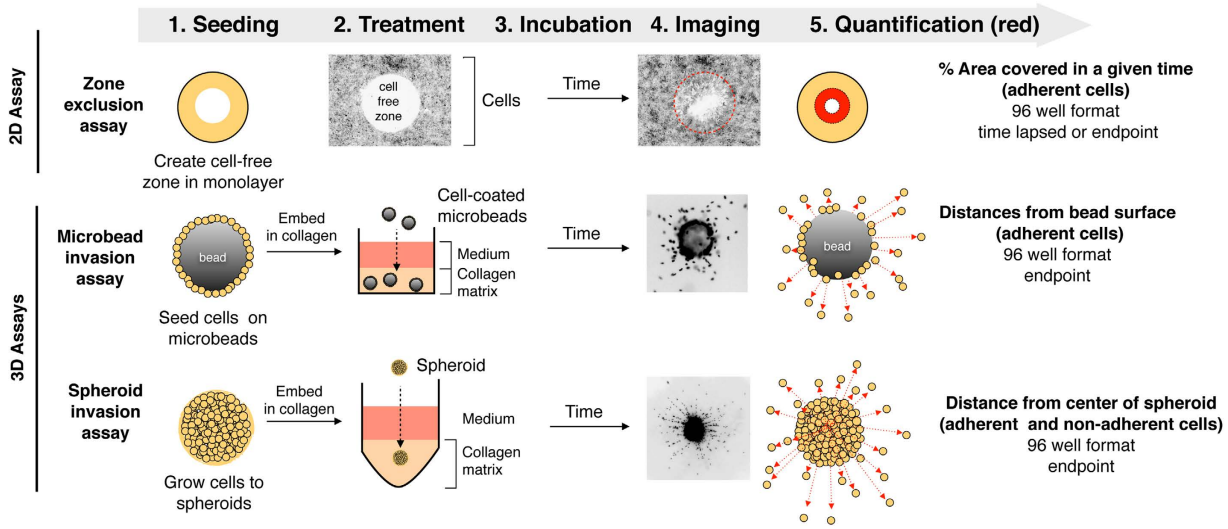


Figure 1. Schematic overview of 2D and 3D cell-based dissemination quantification assays. Cells are depicted as light-brown, filled circles. Alterations in cell localization at a given time point are highlight in red.

cells migrating in 3D are not yet available, mostly because of the difficulties to efficiently measure the distance between origin and endpoint of migration of cells migrating detached from a solid substrate. To enable cell motility quantification in 2D and 3D, we have assembled a package of three cell migration assays and combined them with automated imaging and computational image analysis. This new approach now allows the efficient evaluation of migration-regulating functions of chemical and mechanical cues over a wide range of conditions.

Medulloblastoma (MB) is a highly malignant embryonal neuroepithelial tumour of the cerebellum with a tendency to metastasize within the central nervous system[18]. Genomic analyses classified MB into the four molecular subgroups wingless (WNT), sonic hedgehog (SHH), group 3 and group 4[18,19]. Macroscopic and microscopic evidence of metastases is considered a high risk factor and despite aggressive treatment regimens, one-third of patients succumb to the disease[18]. Metastatic dissemination is specifically associated with tumours of the MB subgroups 3 and 4[18]. However, it can also be triggered in the SHH subgroup by the ectopic expression of selected putative driver genes such as Eras, Lhx1, Ccrk, and Akt[20] or by the activation of growth factor signalling pathways such as hepatocyte growth factor (HGF)-c-Met signalling[21]. The mechanisms triggering and maintaining MB dissemination are largely unknown. We hypothesised that growth factors trigger detachment and dissemination of cells from the primary tumour. Therefore, we tested the migratory response of established SHH MB lines[22], medulloblastoma patient-derived xenograft (Med PDX) and primary MB lines to growth factor stimulation and thereby explored the potential impact of such factors to metastatic dissemination. We show the validation of our automated cell motility analysis platform and demonstrate its efficacy and functionality to determine factors driving the dissemination of both established cancer cell lines and primary tumour cells.

Results

Automated quantification of cell dissemination. To explore extrinsic and cell intrinsic factors controlling collective cancer cell dissemination in 2D, we used the zone exclusion assay[17], which provides circular cell free surfaces of identical area and allows the quantification of area covered by cells over time (Fig. 1). A high-throughput assay was recently developed to quantify the number of cells disseminated from spheroids into collagen I matrix[14]. However, this assay does not allow the quantification of the cell spreading distances. Moreover, the mode of cell migration is semi-3D as the cells are allowed to attach to and migrate on a solid support. To overcome these limitations and to quantify cell dissemination and invasion in 3D, we established the microbeads cell dissemination/invasion and the spheroid cell dissemination/invasion assays. Both assays are true 3D assays that allow the cells to disseminate fully detached from a defined reference point into a 3D matrix. The reference points are the surface of the microbeads and the centres of the spheroid, respectively (Fig. 1). The main difference between the microbeads and the spheroid invasion assay is that former measures dissemination/spreading of cells grown into a two-dimensional monolayer on the bead surface into the collagen matrix (2D to 3D), whereas latter measures dissemination/spreading of cells from three-dimensional cell aggregates (3D to 3D).

The zone exclusion assay works best with cells displaying cytosolic fluorescence (e.g. EGFP, lifeact-EGFP, or fluorescent live cell stains), whereas the 3D assays require nuclear staining for better separation of individual cells. For each assay we developed the corresponding software tools (Fig. 2),

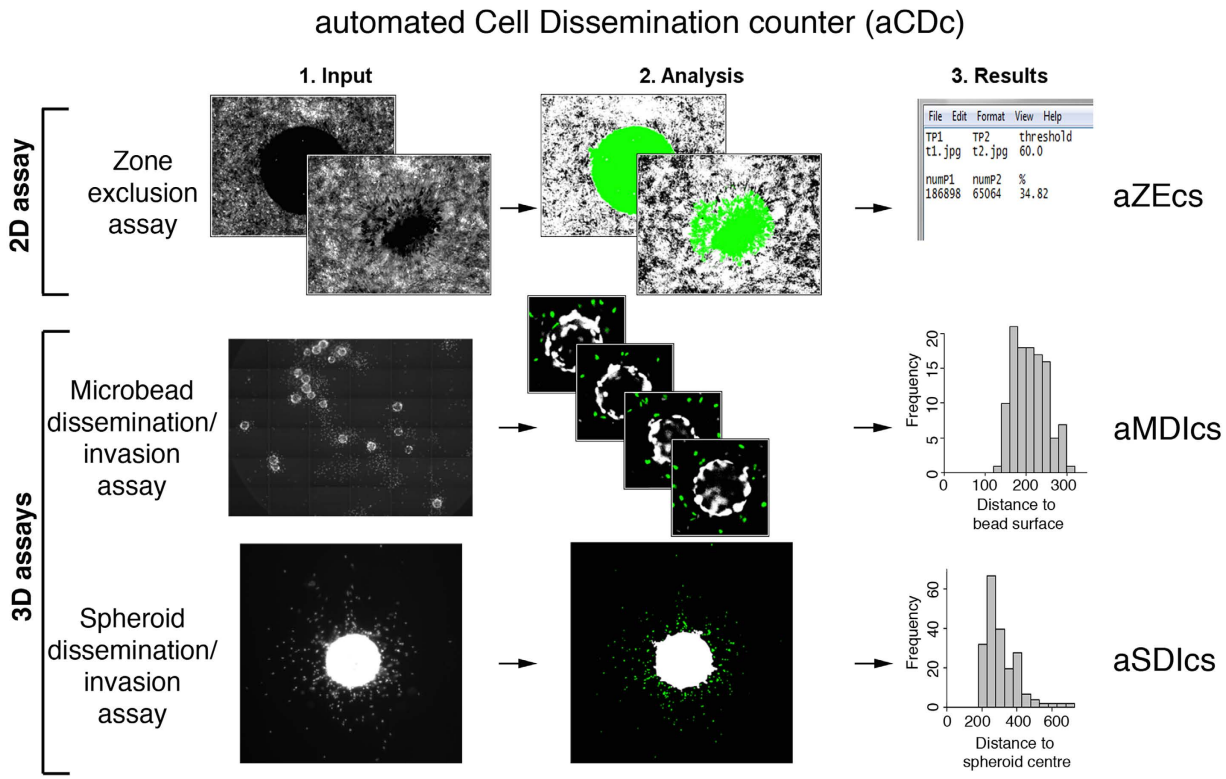


Figure 2. Schematic overview of working principle of 2D and 3D cell dissemination and invasion counter software tools: aZECs (automated Zone Exclusion counter software), aMDIcs (automated Microbeads Dissemination/Invasion counter software) and aSDIcs (automated Spheroid Dissemination/ Invasion counter software). Green area in aZECs represents area recognized and quantified by software and green nuclei highlight the cells recognized and counted by aMDIcs and aSDIcs.

which either progressively quantify the area covered by cells (2D zone exclusion) or number and distance of disseminated cells (3D assays). We named the software according to their working principles: aZECs (automated Zone Exclusion counter software), aMDIcs (automated Microbead Dissemination/ Invasion counter software) and aSDIcs (automated Spheroid Dissemination/Invasion counter software). A program suite referred to as automated Cell Dissemination counter (aCDc) consisting of three open source, executable Java programs (.jar) files can be downloaded through the following web link http://www.infozentrum.ethz.ch/uploads/user_upload/Software/.

Assay Validation. HGF/c-Met signalling promotes motility of MB tumour cells, and is associated with an aggressive invasive phenotype in patients[21,23]. Therefore, we used HGF stimulation to validate our assays in combination with aCDc. We treated the human SHH MB cell lines DAOY and UW228 in the zone exclusion assay with 20 ng/ml HGF in the absence or presence of pharmacological c-Met inhibitors (PHA665752 or ARQ197, 125 nM). Using aZECs, we analysed images acquired under both 10% serum (Fig. 3A–E) and serum-free conditions (Supplementary Fig. S1). Both cell lines stably express lifeact-EGFP (LA-EGFP) for monitoring F-actin dynamics. Consistent with our previous quantifications[23], blocking c-Met with pharmacological inhibitors reduced basal migration and prevented HGF-induced motility (Fig. 3B,C and Supplementary Fig. S1A, B). An inherent problem of the zone exclusion assays is that cell proliferation could falsify motility outputs based on area covered. We therefore used time-lapse video microscopy imaging, which provides refined information on cell dissemination at time points (5–10 h) when proliferation effects can be considered marginal. Indeed, we found that HGF treatment accelerated cell migration significantly (T-test, $p = 0.00014$) already within 5 h incubation under 10% serum (Fig. 3D,E) and serum-free conditions (T-test $p = 0.0276$, Supplementary Fig. S1C, D). Consistently, this pro-migratory effect of HGF was blocked with pharmacological c-Met inhibitors, which caused the reduction of cell migration by 50% within 5 h incubation in DAOY (Fig. 3D, Supplementary Fig. 1C) and UW228 cells (Fig. 3E, Supplementary Fig. 1D).

We used the same cell lines to assess the effects of HGF, PHA665752 and ARQ197 on cell dissemination in the 3D environment. The images were acquired either on a Molecular Device automated microscope (microbeads) or on a Zeiss AxioObserver (spheroids) and quantified using aMDIcs and aSDIcs. We found that treatment with HGF induced extensive invasiveness into collagen, both from

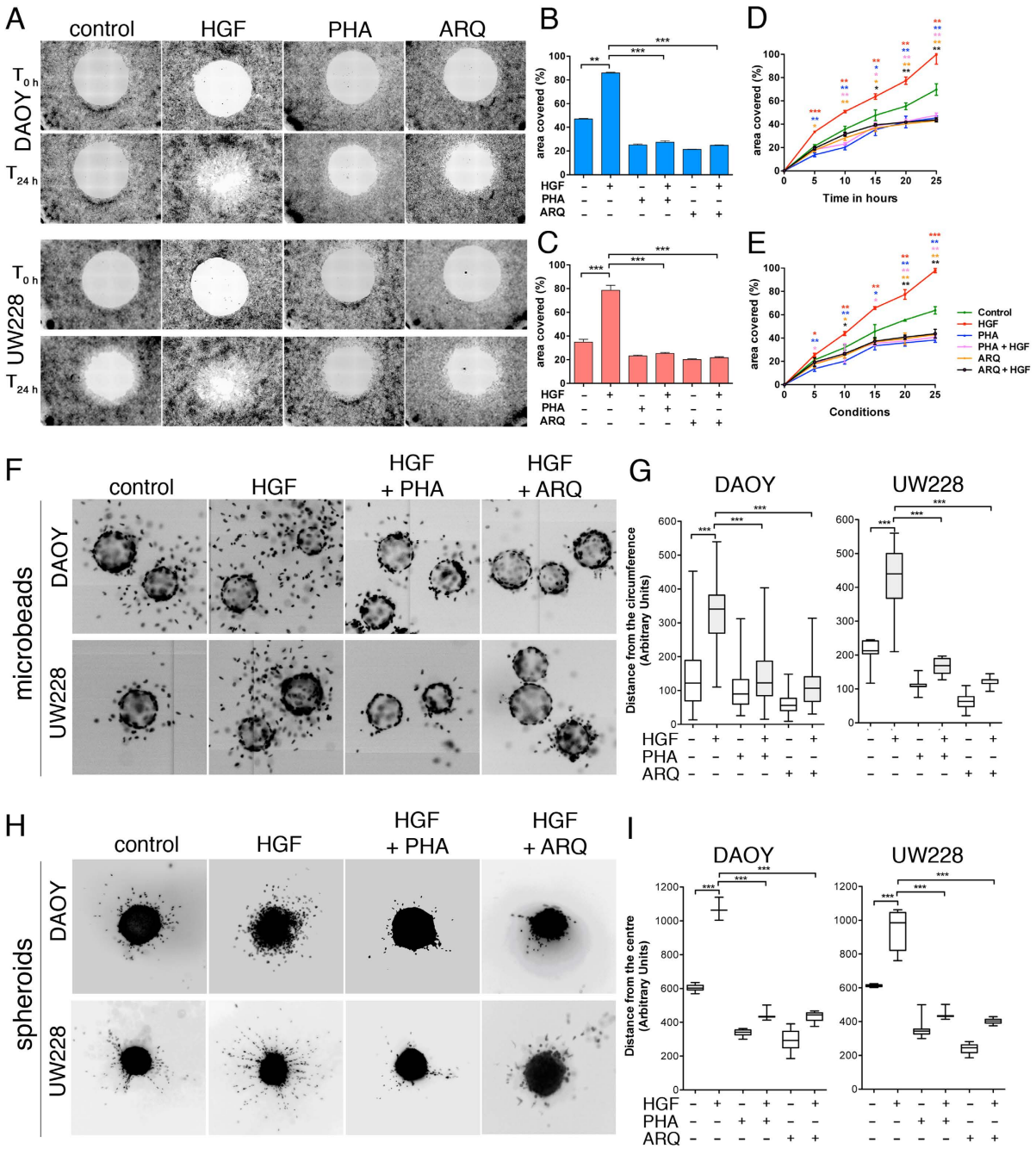


Figure 3. aZEcs, aMDIcs and aSDIcs quantifications reliably confirm manual measurements of HGF-induced cell dissemination in the presence of 10% serum. (**A**) Representative images of 100× magnified zones of exclusion in monolayers of LA-EGFP expressing DAOY or UW228 cells in 96 well plate. Images are inverted greyscale of LA-EGFP fluorescence at 0 (T_{0h}) and 24 h (T_{24h}) −/+ stimulation with 20 ng/ml HGF and/or treatment with c-Met inhibitors PHA665752 and ARQ197 (125 nM each). (**B,C**) Means and SDs of % area covered from three independent zone infiltration experiments using aZEcs in DAOY (**B**) or UW228 (**C**) at T_{24h} after HGF stimulation and/or inhibitor treatment. (**D,E**) Time-lapsed mean and SD quantifications of % area covered from three independent zone infiltration experiments using aZEcs in DAOY (**D**) or UW228 (**E**) after HGF stimulation and/or inhibitor treatment. (**F**) Representative images of 100x magnified microbeads coated with DAOY or UW228 cells after 24 h −/+ stimulation with 20 ng/ml HGF and/or treatment with c-Met inhibitors PHA665752 and ARQ197 (125 nM each). Inverted greyscale images of Hoechst-stained nuclei are shown. (**G**) Quantification of distance from the surfaces of the microbeads using aMDIcs. Box plots with pooled data of two independent experiments and whiskers min to max are shown. (**H**) Spheroid invasion assay with DAOY and UW228 cells. Treatment and imaging as in F), except that 50× magnification was used. (**I**) Quantification of distance from centres of spheroids using aSDIcs. Box plots with pooled data of two independent experiments and whiskers min to max are shown.

microbeads (Fig. 3F,G, Supplementary Fig. S2, S3E Supplementary videos 1–4) and spheroids (Fig. 3H,I, Supplementary Fig. S3F), which was abolished with concomitant PHA665752 or ARQ197 treatments.

Overall, we show that the motility assays developed, in combination with aCDc, provide versatile end-point or time-lapse quantification means that can rapidly and accurately measure cell migration and invasiveness.

Differential impact of growth factors and cytokines on MB cell migration and invasiveness. Growth factors in the tumour microenvironment play a critical role in tumour initiation, progression and metastasis[24]. Therefore, we determined the effect of predominant growth factors/cytokines present in the tumour microenvironment of MB on cell migration and invasiveness. We analysed the effect of HGF (20 ng/ml), EGF (30 ng/ml), IGF (20 ng/ml), netrin (200 ng/ml), PDGF-B (20 ng/ml), TNF-α (25 ng/ml), bFGF (100 ng/ml), IL-6 (20 ng/ml), NGF (50 ng/ml), TGF-β (20 ng/ml) and PlGF-1 (10 ng/ml) using the 2D zone exclusion assay and quantified it with aZECs. HGF, EGF and bFGF significantly promoted cell migration in DAOY and UW228 cells under both 10% serum and serum free conditions (One-way ANOVA $p \leq 0.05$). IL-6, IGF and PlGF-1 moderately influenced cell migration while the other factors did not significantly impact on cell migration (Fig. 4A,B and Supplementary Fig. S3A, B). Based on the above results, we were able to classify the growth factors and cytokines as strong, moderate and weak promoters of cell migration. Using time-lapse video microscopy, we found that bFGF promoted cell migration significantly within 15 h in DAOY cells (T-test, $p = 0.0026$), while IGF's effect was significant (T-test $p = 0.0197$) only after 20 h (Fig. 4C and Supplementary Figure S3C). EGF and IL-6 significantly increased (T-test, $p = 0.00741$ and 0.0136, respectively) UW228 cell migration after 10 h of incubation compared to untreated control (Fig. 4D and Supplementary Fig. S3D).

To better understand the relationship between tumour microenvironment parameters and cell invasion, we studied the effects of HGF, EGF, IGF, netrin, PDGF-B, TNF-α, bFGF, IL-6, NGF, TGF-β and PlGF-1 in the 3D environment. We found that the signature pattern of promoting cell migration in 2D environment as strong, moderate and weak was conserved in the 3D environment. HGF, EGF and bFGF induced cell invasiveness in both microbead invasion and spheroid invasion assay under 10% FCS (Fig. 4E,F) and serum-free conditions (Supplementary Fig. S3E, F). Precise quantification using aMDIcs and aSDIcs showed that IGF had a moderate influence on cell invasion of DAOY but not of UW228 cells (Fig. 4E,F, Supplementary Fig. S3E, F). Similarly, IL-6 had a moderate influence on cell invasion of UW228 but not of DAOY cells (Fig. 4E,F, Fig. Supplementary Fig. S3E, F), depicting the differential behaviour of the two cell lines with similar phenotypes.

Taken together, we showed that there are strong, moderate and weak promoters of cell dissemination and invasion in the 2D and 3D environment and that each cell type responds differently to these stimuli. The distinct reactions of each cell type to different stimuli can be accurately identified and quantified using aCDc.

Evaluation of sub group-specific sensitivities to promigratory stimuli. Differences in sensitivity to growth factor-induced cell motility between the closely related DAOY and UW228 lines implied that cells of individual tumours might respond differently to growth factor stimuli. We therefore tested whether patients could be stratified based on growth factor sensitivity, ultimately for adapting therapy schemes to growth factor sensitivities of the tumor. Therefore, we analysed the effects of the various growth factors/cytokines on the primary line ZH-513, which we established from a non-SHH/WNT MB tumour sample (Fig. 5A). Of note, HGF, EGF and bFGF had the maximum influence on cell invasiveness (Fig. 5B), similar to that observed in DAOY and UW228. However, we observed significant differences (One-way ANOVA $p \leq 0.0001$) in the cell motility of ZH-513 cells when stimulated with NGF, PlGF-1 and TGF-β compared to DAOY (Fig. 4F) or Med PDX 1712 cells (Fig. 5C), indicating a difference in sensitivity and/or response among MB sub-groups. To check whether the same signature of growth factor-induced cell invasion is maintained in the ZH-513 cells, we repeated the same experiment after 10 days of culturing the primary cells *in vitro* (Fig. 5B, trial 2). The ZH-513 cells maintained their signature pattern of cell invasiveness with HGF, EGF and bFGF being strong promoters of invasiveness and IL-6, NGF, PlGF-1 and TGF-β being intermediate ones (Fig. 5B). These results demonstrate that primary patient material can be grown *in vitro* and tested using the spheroid invasion assay and aSDIcs for efficient diagnosis.

To further confirm the usability of aSDIcs for diagnostic evaluation of cell dissemination, we studied the impact of growth factors and cytokines on the MB Med PDX cell lines Med 1712 and Med 411. Invariably, HGF, EGF and bFGF had a strong influence on cell dissemination in both PDX lines (Fig. 5C,D). Differences between the two lines further substantiate the fact that response to growth factors can be used to identify aberrantly activated signalling pathways. IGF and IL-6 significantly increased (One-way ANOVA, $p \leq 0.05$ and 0.01, respectively) cell dissemination in Med 1712, (Fig. 5C), while IL-6, NGF and PlGF-1 did so in Med 411 (One-way ANOVA, $p \leq 0.05$, Fig. 5D) compared to untreated controls. Direct comparison of the relative impact of all factors between Med 1712 and Med 411 also revealed significant differences when the cells were stimulated with either NGF or PlGF-1 (One-way ANOVA, $p \leq 0.0001$ and 0.05, respectively, Fig. 5E). The results above indicate that whether or not the cells respond to NGF and PlGF-1 may be used as markers to differentiate the two PDX cell lines and likely also other SHH and group 3-derived MB tumour cells.

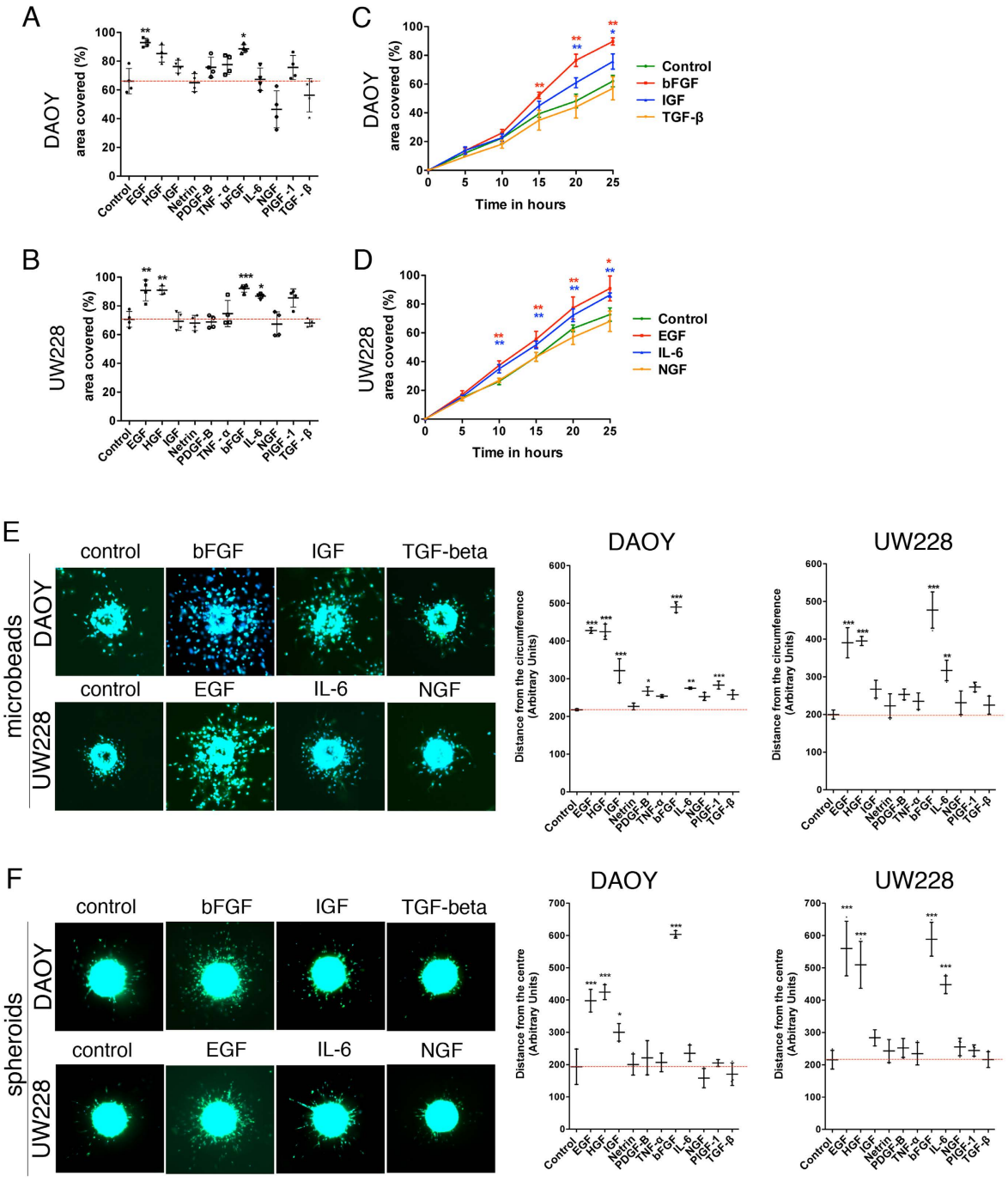


Figure 4. Selective induction of cell dissemination with growth factors in the presence of 10% FCS. (**A,B**) End-point quantification of % area covered in zone infiltration assay using aZEcs. Means and SDs of three independent experiments with DAOY (**A**) or UW228 (**B**) cells at T_{24h} after stimulation with factors as indicated are shown. Concentrations of growth factors/cytokines were as follows: HGF (20 ng/ml), EGF 30 ng/ml), IGF (20 ng/ml), netrin (200 ng/ml), PDGF-B (20 ng/ml), TNF-α (25 ng/ml), bFGF (100 ng/ml), IL-6 (20 ng/ml), NGF (50 ng/ml), TGF-β (20 ng/ml) and PlGF-1 (10 ng/ml) (**C,D**) Time-lapsed mean and SD quantifications of % area covered from three independent zone infiltration experiments using aZEcs in DAOY (**C**) or UW228 (**D**) cells stimulated with factors as indicated. (**E**) Representative images of 100× magnified microbeads coated with DAOY or UW228 cells after 24 h −/+ stimulation with bFGF, IGF or TGF-beta (DAOY) or EGF, IL-6 or TGF-beta (UW228). LA-EGFP in green, Hoechst staining in blue. Right panels: quantification of means and SDs of cell dissemination/invasion from three independent experiments using aMDIcs. Concentrations of growth factors/cytokines as in 4(**A,B**). (**F**) Representative images of 50× magnified spheroids of DAOY or UW228 cells after 24 h −/+ stimulation with bFGF, IGF or TGF-beta (DAOY) or EGF, IL-6 or NGF (UW228); LA-EGFP in green, Hoechst staining in blue. Right panels: Quantification of distance of cell dissemination/invasion using aSDIcs. Concentrations of growth factors/cytokines as in 4(**A,B**).

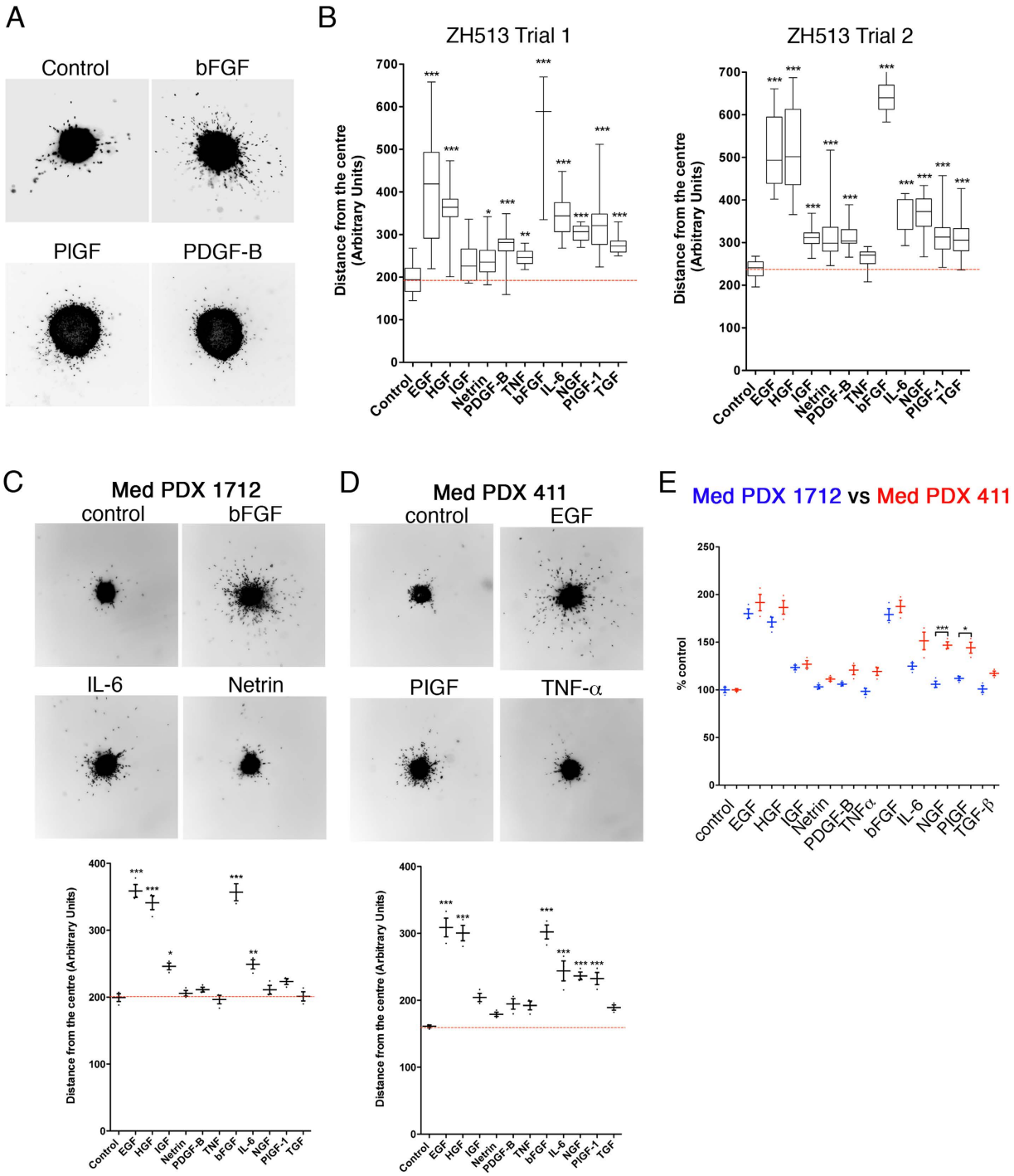


Figure 5. Quantification of growth factor-induced dissemination/invasion of primary MB tumour cells. (**A**) Representative images of 50× magnified spheroids of primary MB tumour cells after 24 h −/+ stimulation with bFGF, PlGF and/or PDGF-beta. Inverted greyscale images of Hoechst-stained nuclei are shown. (**B**) Quantification of distance from centres of spheroids in two experimental replicas using aSDIcs. Concentrations of growth factors/cytokines as in 4(**A**,**B**). Box plots with whiskers min to max are shown. Representative images of 50× magnified spheroids of Med PDX 1712 (**C**) and Med PDX 411 (**D**) and the quantification of average cell disseminations using aSDIcs. Concentrations of growth factors/cytokines as in 4A,B. Mean and S.D. of three experiments are shown. (**E**) Dot plot compares average dissemination of Med PDX 1712 (blue) and Med PDX 411 (red) relative to unstimulated controls.

Collectively, we have revealed similar and differential growth factor responses of primary patient material and PDX cell lines compared to the established MB cell lines. These different migratory responses of the samples to growth factor stimulation can be easily detected and quantified using aSDIcs and may be

exploited to sub-group patient samples and PDX cell lines based on their sensitivities to growth factor stimuli.

Discussion

We have developed an assay platform to quantify cancer cell dissemination in 2D and 3D environments in high-throughput. This platform consists of cell-based assays, imaging devices for acquisition and software solutions for the quantification of the imaging data. Importantly, aCDc saves the original and the processed images as log files, which can always be traced back to confirm appropriate quantification. aZEcs takes into account pipetting artefacts and eliminates the deficits before quantification, a feature which is not found in any other available software. We validated the assay platform using the pro-migratory growth factor HGF in a cell-based model of SHH-MB. Using aCDc, we furthermore determined for the first time the migratory response profile of two long-term, one primary and two patient-derived xenograft human MB lines to a selection of eleven growth factors and cytokines with described or suspected micro-environmental impact on MB growth and dissemination.

One limitation of 2D assays is that the invasive capability of the cells cannot be addressed adequately, even when adherent cells are embedded in 3D matrix. This is due to marked differences in cells adherent to a rigid surface compared to fully embedded cells with respect to cell signalling, motility modes and plasticity of migration[25] (and discussed and referenced in[26]), which are critical parameters for tumour cell dissemination *in vivo* as well[27]. Hence, in addition to the zone exclusion assay, we used 3D assays to explore steady state and induced motile MB cancer cell behaviour and to identify growth factors relevant for matrix invasion and dissemination. Overall, our analyses showed a good consistency between 2D and 3D assay data for strong promoters of cell dissemination such as HGF, EGF and bFGF. However, steady-state migration was markedly higher in 2D than in 3D while differences between treatments were subtler. In contrast, and particularly striking for the spheroids, steady-state migration is restricted in 3D and requires a stimulus. This indicates that MB cells on a stiff substrate such as glass or plastic migrate spontaneously into cell free areas and suggests that the molecular mechanisms underlying motility control differ between 2D and 3D conditions in these cells.

Time-lapse movies indicated that cell movement in 3D occurs mostly outward with respect to the surface of the bead or the spheroid (videos 1–6). Mean distances between cells and beads/spheroids calculated based on the projected images using aMDIcs and aSDIcs correspond approximately to the expected effective distances, providing their position is within a 25° angle above or below the horizontal axis (Fig. S4). The quantified distance between cells and beads/spheroids outside this angle is underestimated proportional to the respective angle between cell, bead/spheroid surface and horizontal axis (Fig. S4B). However, as the depth of field is centered on the horizontal axis, the vast majority of objects analyzed by the software are within 25° off the horizontal axis and the underestimation error equal under all conditions can be considered marginal.

When aZECs is used in combination with mitogenic factors such as HGF, we recommend a time-lapsed analysis of the progression of zone exclusion, to minimize proliferation-dependent artefacts by selecting time points when proliferation effects can be considered marginal. Proliferation is less problematic in the 3D assays, as aMDIcs and aSDIcs measure distances and automatically count the number of disseminated cells. aMDIcs yielded higher variance in the distances of invasion in individual DAOY samples compared to UW228 cells. We ascribe these differences to the cell-specific capability to adhere to the cytodex microcarrier beads, which is greater for UW228 cells and appears to increase the threshold of pro-migratory cues necessary to promote detachment and dissemination. No pronounced difference in distance of migration was observed, indicating that the mode of cell growth prior to migration – spheroid versus monolayer on bead - is not determining the degree of response in responsive MB cells. However, cells in spheroids remained clustered even after treatment with bFGF, which overall triggers massive dissemination. Hence, only a subset of cells responds to growth factors with dissemination in spheroids, while the majority of cells disseminate from microbeads. What restricts spheroid cell dissemination is unknown but it is possible that cell-cell contact, which is tight in spheroids and relatively loose on microbeads, is an additional determinant for the migratory outcome. The morphodynamic processes underlying cell dissemination from spheroids or microbeads can be further addressed using lifeact-EGFP expression, which allows visualizing F-actin dynamics in disseminating cells in real time[28] (Videos 1–6). Live cell imaging combined with confocal microscopy of MB cells migrating from microbeads (Fig. S2), indicated that dissemination relies on mesenchymal migration. This migration appears to be driven by F-actin-rich, dynamic protrusions at the leading edge of the migrating cells and supported by matrix remodelling (collagen fibre tethering (S2Aa), collagen degradation (S2Ab) and tunnel formation (S2Bc)).

Genomic analyses of large cohorts of MB now allow discriminating four molecular subgroups with defined molecular, functional and clinical characteristics[18,29]. We propose here a complementary method of MB subgroup characterisation that is based on the growth factor sensitivity profiles of the tumours. Although refinement will be needed and larger sample sizes necessary for standardization, this method may enable the rapid diagnostic evaluation of cells extracted from the primary tumour. Indeed, we can discriminate the established laboratory lines and the SHH PDX line Med 1712 from the non-WNT/SHH line ZH-513 and the group 3 PDX line Med 411, as latter two display selective sensitivities to NGF and PIGF-1. In addition, the high throughput capabilities of our platform in combination with primary or PDX material permit efficient co-clinical testing of potential anti-metastatic drugs. Since the assay

is based on cell dissemination triggered by effective signalling through the relevant receptors and their underlying signalling pathways, it also reveals the growth factor sensitivity of a given tumour. Unlike gene expression data, this functional, co-clinical assessment of patient-derived material may provide biologically relevant information on the tumour. An example is PlGF-1 signalling, which along with its receptor neuropilin 1 is expressed in the majority of human MB and specifically contributes to growth and spread of group 3 tumours[30]. Our data in the non-WNT/SHH line ZH-513 and the group 3 PDX line Med 411 now support the notion of the specific, pro-tumorigenic impact of PlGF-1 in non-WNT/SHH MB. They furthermore indicate a pro-migratory function of PlGF-1 in MB that may explain reduced metastasis observed in orthotopic group 3 and group 4 murine models of human MB after PlGF-1 blockade[30].

Targeting cell motility alone might not be sufficient to eradicate the cancer; however, it may contribute to its control by restricting local infiltration, by limiting further dissemination and by preventing the evolution towards a more aggressive phenotype[3]. HGF[21,23,31,32] and EGF[33] were previously shown to promote motility of MB cells. No pro-migratory functions of bFGF have been described in MB so far. In contrast, bFGF was found to interfere with SHH signalling in neuronal precursor and tumour cells[34] and to block tumour formation in a mouse model of SHH MB[35]. Thus, although bFGF treatment elicits anti-tumorigenic responses in susceptible cell lines, its dissemination promoting functions calls for caution when bFGF treatment is considered as therapeutic strategy. HGF, EGF and bFGF activate receptor tyrosine kinases that likely feed into the same downstream signalling pathways. Therefore, an ideal target for a future drug would be a molecular hub that integrates signals from all three pathways. With MAP4K4 we recently identified such a potential convergence kinase downstream of HGF function in MB[23], which is also activated by EGF[36] and TNFa[37] to promote cell motility. aCDc also identified the specific response of UW228 cells to IL-6 stimulation. Hence, in this case, an ideal target would be an effector that orchestrates HGF, EGF, bFGF and IL-6 pathways. This implicitly proves that aCDc may be used to identify specific differences among the cell types, which could be exploited to effectively target the relevant pathways.

In conclusion, we provide a novel method of cancer cell evaluation according to their growth factor-dependent dissemination behaviour. We expect this method to improve accuracy of diagnosis and ultimately aid in the tumour-specific refinement of treatment schemes for patients, to increase cure rates and to reduce treatment related morbidities.

Methods

Ethics Statement. Informed consent was obtained from subjects and all research involving subject's material was conducted under appropriate review/privacy board protocols of the Kantonale Ethikkommission Zürich (Ethics Commission of the Canton of Zürich, Switzerland). The use of patient tumour material for diagnostic and prognostic analysis was approved by the Kantonale Ethikkommission Zürich.

Reagents. Growth factors and inhibitors were used throughout the study in the concentrations indicated. Hepatocyte growth factor (HGF): 20 ng/ml (100-39), epidermal growth factor (EGF): 30 ng/ml (100-47), Insulin like Growth Factor 1 (IGF): 20 ng/ml (100-11), Netrin: 200 ng/ml (R&D Systems, 6419-N1-025), Platelet Derived Growth Factor-B (PDGF-B): 20 ng/ml (P100-14B), Tumour Necrosis Factor-α (TNF- α): 25 ng/ml (300-01A), basic Fibroblast Growth Factor (bFGF): 100 ng/ml (100-18B), Interleukin-6 (IL-6): 20 ng/ml (200-06), Nerve Growth Factor-β (NGF): 50 ng/ml (450-01), Transforming Growth Factor-β (TGF-β): 20 ng/ml (100-21) and Placental Growth Factor-1 (PlGF-1): 10 ng/ml (100-06) from PeproTech (London, UK), PHA-665752, 125 nM (Selleckchem, Houston, TX, USA, S1070), ARQ197, 125 nM (Active Biochemicals, Wanchai, Honkong, A-1109).

Human MB cell lines, human primary MB cell and patient-derived xenograft (PDX) culture. DAOY human MB cells (originally derived from a desmoplastic cerebellar MB) were purchased from the American Type Culture Collection (ATCC, Rockville, MD, USA). UW228[38] was generously provided by John Silber (Seattle, USA). DAOY and UW228 cells were cultured as described in[39]. DAOY LA-EGFP and UW228 LA-EGFP cells were produced by lentiviral transduction of DAOY and UW228 cells with pLenti-LA-EGFP[23]. Human primary medulloblastoma cells were derived from a resected tumour of a nine-year-old patient with an M2 stage medulloblastoma with cerebellar metastases. Histology indicated non-SHH, non-WNT type[40]. Tumour tissue was acutely dissociated using a papain-based dissociation kit (Worthington Biochemical, Lakewood, NJ) according to the manufacturer's protocol. Following dissociation, cells were cultured in neurobasal medium (Invitrogen/Life Technologies, Paisley, UK, 12349-015) supplemented with 2% B-27® (Gibco/Life Technologies, 10889-038), 1% L-Glutamine (Invitrogen/Life Technologies, 25030024), 10 μg/ml bFGF and 10 μg/ml EGF. The human SHH PDX line Med-1712FH and the Group3 line Med-411FH were obtained from the brain tumour resource laboratory of the Fred Hutchinson Cancer Center and maintained in the same growth medium as the primary tumour cell.

Zone infiltration assay. Oris™ 96-well cell seeding stoppers were inserted in μ-clear 96 well plate (Greiner CELLSTAR®, Frickenhausen, Germany, 655090) and 3.5×10^4 cells/well were seeded. Cells were incubated at 37 °C overnight to form the zone of exclusion. The following day, after plug removal, the

cells were treated with growth factors/cytokines or with HGF and PHA-665752 or ARQ197. Cell migration was monitored for 25 h using an automated ImageXpress Micro 2 microscope (Molecular Devices, LLC, USA) equipped with environmental control. Arrays of 5×5 images per well were acquired at 5 h intervals with a 10×0.2 NA Plan Apo objective (Nikon) and a Roper CoolSNAP HQ camera (Roper Scientific, Martinsried, Germany). Cell migration was measured as percentage of cell free area covered by the cells in a given time (time lapsed or endpoint) using the automated computer-assisted cell migration quantification software aZEcs.

Microbead invasion assay. Approximately 500 Cytodex Microcarrier beads (Sigma Aldrich, St. Louis, MO, USA, C3275) per 1.25×10^4 cells/ml were mixed in FACS tubes (Becton, Dickinson and Company, Allschwill, Switzerland, Falcon T7597-5J) and incubated at 37 °C for 6 h with a mild shaking of the tubes at every one hour. Cells, which were not adhered to the beads, were removed by washing the beads with fresh medium. Cell-coated microbeads were re-suspended in 2.5% bovine collagen 1 (Advanced BioMatrix, San Diego, CA, USA, 5005-B) and seeded in μ-clear 96 well plate (Greiner CELLSTAR®, 655090). Following the polymerization of collagen, the cell coated microbeads were overlaid with fresh medium and treated with appropriate concentrations of growth factors/cytokines or with HGF or with c-Met inhibitors. After 24 h, cells were fixed with 4% paraformaldehyde (PFA) and stained with Hoechst. Images were acquired using an ImageXpress Micro 2 automated microscope (Molecular Devices, LLC, USA) as described for the zone infiltration assay. Cell invasion is expressed as the average of the distance invaded by the cells from the circumference of the bead as measured by our cell dissemination counter software aMDIcs.

Spheroid invasion assay. 1000 cells/100 μl per well were seeded in a 96 well Lipidure®-Coat plate A-U96 (Amsbio, Bioggio-Lugano, Switzerland, AMS.51011610). The cells were incubated at 37 °C overnight to form spheroids. 70 μl of the medium were removed from each well, and remaining medium with spheroid was overlaid with 2.5% bovine collagen 1. Following the polymerization of collagen, fresh medium was added to the cells and treated with growth factors/cytokines or with HGF and/or c-Met inhibitors. The cells were allowed to invade the collagen matrix for 24 h, after which they were fixed with 4% PFA and stained with Hoechst. Images were acquired on an Axio Observer 2 mot plus fluorescence microscope (Zeiss, Munic, Germany) using a 5× objective. Cell invasion is determined as the average of the distance invaded by the cells from the center of the spheroid as determined by our cell dissemination counter software aSDIcs.

Live imaging. *Microbead invasion assay.* Cytodex microcarrier beads were coated with cells according to the protocol described above. The cell-coated microbeads re-suspended in collagen were seeded in 8 well ibidi plate (ibidi, Martinsried, Germany 80821). After collagen polymerization, the cell coated microbeads were overlaid with fresh medium and treated with appropriate concentrations of growth factors/cytokines. Cell invasion was monitored for 18 h using a Axio Observer 2 mot plus fluorescence microscope (Zeiss). Images were acquired using a 20× objective at the interval of 30 minutes and the time point images were assembled into a QuickTime video (5 fps).

Spheroid invasion assay. 500 cells/40 μl per well were seeded in a Perfecta3D® 96 well hanging drop plate (3D Biomatrix, Ann Arbor, MI, USA, HDP1096) according to the manufacture's instructions and incubated at 37 °C for 72 h to form spheroids. The spheroids were harvested onto an 8 well ibidi plate (one spheroid per well) via the top access hole method as described by the manufacturer. The spheroids were overlaid with 2.5% bovine collagen 1. Following the polymerization of collagen, fresh medium was added to the cells and treated with growth factors/cytokines. Cell invasion monitoring and assembly of the video was performed similar to microbead invasion assay (live imaging) protocol.

Software development. *aZEcs.* For automatic evaluation of zone exclusion, the algorithm determines the percentage of a cell-free area at time point T_1 that remains uncovered by cells at a later time point T_2. In the first step, the microscopy images are converted into dichroic black and white images according to a RGB threshold, where white fields correspond to areas covered by cells. This RGB threshold is calculated individually for every image based on the user-defined parameter *rgbt*, which represents the percentage of white pixels after conversion. By adjusting this parameter, the user is able to account for over- and underexposed images. Due to the high content of visible cells in the images, the default setting for the zone exclusion assay is $rgbt = 60$. In the second step, a clustering algorithm determines the size of the cell-free area in the images recorded at T_1. Starting with a single black pixel, all adjacent black pixels are joined to form a cluster, and this procedure is recursively continued until no additional pixel can be added. Then, the next black pixel not yet belonging to a cluster is chosen and processed until a set of clusters is obtained. The cluster with the maximum area found in this procedure is defined as the cell-free area. The remaining cell-free area at time point T_2 is expressed as the percentage of cell-free area at T_1.

aMDICs. For automatic evaluation of cell motility using the microbeads dissemination/invasion assay, aMDIcs first performs bead identification. The acquired image is again converted to black and white as for the zone exclusion assay. Due to the reduced number of cells compared to the zone exclusion assay,

the default value for the dichroic conversion is $rgbt = 1$. A naïve brute force algorithm then systematically scans the whole image for circular, white structures. It does so by shifting a growing circle over the image from left to right and from top to bottom. For every position and diameter, the algorithm calculates the percentage of the circle that is covered by white pixels in the dichroic image. All objects that cover more than a user-defined fraction (default = 50%) of the circle with white pixels are considered as a bead. For these objects, the algorithm stores the centre of the circle and the respective diameter. After scanning the image, the algorithm recursively selects the beads with the highest coverage and extracts them from the original image for analysis. This is done to ensure that the respective local optimum (in terms of bead centre and diameter) is chosen for every bead. Then the bead-to-bead distances are calculated and only isolated beads without potentially overlapping migration areas are kept. Cells are identified using the clustering procedure described in the aZEcs algorithm. For aMDIcs and aSDIcs, the neighbourhood definition during the clustering process is user-defined and may allow black pixels in between white clusters in order to detect scattered cells as single clusters. A user-defined minimal and maximal cluster size corresponding to the average area of a single nucleus is applied to identify individual cells. All clusters smaller than the minimum value are considered as debris and removed. Clustered pixels covering an area larger than the defined maximum value are considered as clustered cells. The area of such a cluster is divided by the average area of all nuclei analysed, yielding the approximate number of cells contributing to that cluster. All clusters containing white pixels adjacent to the bead surface are removed, as these pixels derive from cells that have not migrate away from the bead. For the remaining clusters, the mean distance between all pixels and the bead centre is calculated. A histogram depicting the cumulated means is generated (Fig. 2) and a log-file, containing the means and standard deviations is produced. For aMDIcs, an additional histogram with the combined data of all located beads is generated to depict variations between beads.

aSDIcs. For automatic evaluation of cell motility using the spheroid dissemination/invasion assay, the images are also converted into a dichroic image. White pixels in close proximity are then clustered and processed as described for aMDIcs. In contrast to the microbeads dissemination/invasion assay, in the spheroid invasion assay no bead surface is available as reference point. The spheroid is therefore converted into a single cluster of white pixels and the centre of this cluster defines the reference point for distance quantification.

Statistical analysis. Mean ± SD are shown when means of three independent experiments are compared, and box plots with whiskers to min and max are shown when multiple individual measurements from three independent experiments (except Fig. 5B) are compared. Unpaired student's t-test was used to test significance of differences between two samples in zone infiltration experiments (Fig. 3B–E and S1). For all other analyses one-way ANOVA repeated measures test using Bonferroni's Multiple Comparison was performed. *P*-Values < 0.05 were considered significant. ($^{*}p \leq 0.05$, $^{**}p \leq 0.01$, $^{***}p \leq 0.0001$). Where indicated, asterisks show statistical significances between control and test sample.

References

1. Comerford, I., Kara, E. E., McKenzie, D. R. & McColl, S. R. Advances in understanding the pathogenesis of autoimmune disorders: focus on chemokines and lymphocyte trafficking. *British journal of haematology.* **164,** 329–341 (2014).
2. Sokol, C. L. & Luster, A. D. The chemokine system in innate immunity. *Cold Spring Harbor perspectives in biology.* **7,** doi: 10.1101/cshperspect.a016303 (2015).
3. Wells, A., Grahovac, J., Wheeler, S., Ma, B. & Lauffenburger, D. Targeting tumor cell motility as a strategy against invasion and metastasis. *Trends in pharmacological sciences.* **34,** 283–289 (2013).
4. Sanz-Moreno, V. & Marshall, C. J. The plasticity of cytoskeletal dynamics underlying neoplastic cell migration. *Curr Opin Cell Biol.* **22,** 690–696 (2010).
5. Nurnberg, A., Kitzing, T. & Grosse, R. Nucleating actin for invasion. *Nat Rev Cancer.* **11,** 177–187 (2011).
6. Doyle, A. D., Petrie, R. J., Kutys, M. L. & Yamada, K. M. Dimensions in cell migration. *Curr Opin Cell Biol.* **25,** 642–649 (2013).
7. Rottner, K. & Stradal, T. E. Actin dynamics and turnover in cell motility. *Curr Opin Cell Biol.* **23,** 569–578 (2011).
8. Chhabra, E. S. & Higgs, H. N. The many faces of actin: matching assembly factors with cellular structures. *Nat Cell Biol.* **9,** 1110–1121 (2007).
9. Kaksonen, M., Toret, C. P. & Drubin, D. G. Harnessing actin dynamics for clathrin-mediated endocytosis. *Nat Rev Mol Cell Biol.* **7,** 404–414 (2006).
10. Palmer, T. D., Ashby, W. J., Lewis, J. D. & Zijlstra, A. Targeting tumor cell motility to prevent metastasis. *Advanced drug delivery reviews.* **63,** 568–581 (2011).
11. Kramer, N. *et al. In vitro* cell migration and invasion assays. *Mutation research*. **752,** 10–24 (2013).
12. Zimmermann, M., Box, C. & Eccles, S. A. Two-dimensional vs. three-dimensional *in vitro* tumor migration and invasion assays. *Methods Mol Biol.* **986,** 227–252 (2013).
13. Hulkower, K. I. & Herber, R. L. Cell migration and invasion assays as tools for drug discovery. *Pharmaceutics*. **3,** 107–124 (2011).
14. Evensen, N. A. *et al.* Development of a high-throughput three-dimensional invasion assay for anti-cancer drug discovery. *PLoS One.* **8,** e82811, doi: 101371/journal.pone.0082811 (2013).
15. Chua, K. N. *et al.* A cell-based small molecule screening method for identifying inhibitors of epithelial-mesenchymal transition in carcinoma. *PLoS One.* **7,** e33183, doi: 101371/journal.pone.0033183 (2012).
16. Lemmo, S., Nasrollahi, S. & Tavana, H. Aqueous biphasic cancer cell migration assay enables robust, high-throughput screening of anti-cancer compounds. *Biotechnology journal*. **9,** 426–434 (2014).
17. Gough, W. *et al.* A quantitative, facile, and high-throughput image-based cell migration method is a robust alternative to the scratch assay. *Journal of biomolecular screening.* **16,** 155–163 (2011).
18. Rusert, J. M., Wu, X., Eberhart, C. G., Taylor, M. D. & Wechsler-Reya, R. J. SnapShot: Medulloblastoma. *Cancer Cell.* **26,** 940–940 e941 (2014).

19. Northcott, P. A. *et al.* Medulloblastoma Comprises Four Distinct Molecular Variants. *Journal of Clinical Oncology.* **29,** 1408–1414 (2011).
20. Mumert, M. *et al.* Functional genomics identifies drivers of medulloblastoma dissemination. *Cancer Res.* **72,** 4944–4953 (2012).
21. Faria, C. C. *et al.* Foretinib is effective therapy for metastatic sonic hedgehog medulloblastoma. *Cancer Res.* **75,** 134–146 (2015).
22. Triscott, J. *et al.* Personalizing the treatment of pediatric medulloblastoma: Polo-like kinase 1 as a molecular target in high-risk children. *Cancer Res.* **73,** 6734–6744 (2013).
23. Santhana Kumar, K. *et al.* The Ser/Thr kinase MAP4K4 drives c-Met-induced motility and invasiveness in a cell-based model of SHH medulloblastoma. *SpringerPlus.* **4,** 19, doi: doi:10.1186/s40064-015-0784-2 (2015).
24. Joyce, J. A. & Pollard, J. W. Microenvironmental regulation of metastasis. *Nat Rev Cancer.* **9,** 239–252 (2009).
25. Tozluoglu, M. *et al.* Matrix geometry determines optimal cancer cell migration strategy and modulates response to interventions. *Nat Cell Biol.* **15,** 751–762 (2013).
26. Friedl, P., Sahai, E., Weiss, S. & Yamada, K. M. New dimensions in cell migration. *Nat Rev Mol Cell Biol.* **13,** 743–747 (2012).
27. Giampieri, S. *et al.* Localized and reversible TGFbeta signalling switches breast cancer cells from cohesive to single cell motility. *Nat Cell Biol.* **11,** 1287–1296 (2009).
28. Riedl, J. *et al.* Lifeact: a versatile marker to visualize F-actin. *Nature methods.* **5,** 605–607 (2008).
29. Taylor, M. D. *et al.* Molecular subgroups of medulloblastoma: the current consensus. *Acta neuropathologica.* **123,** 465–472 (2012).
30. Snuderl, M. *et al.* Targeting placental growth factor/neuropilin 1 pathway inhibits growth and spread of medulloblastoma. *Cell.* **152,** 1065–1076 (2013).
31. Onvani, S. *et al.* Molecular genetic analysis of the hepatocyte growth factor/MET signaling pathway in pediatric medulloblastoma. *Genes, chromosomes & cancer.* **51,** 675–688 (2012).
32. Guessous, F. *et al.* Cooperation between c-Met and focal adhesion kinase family members in medulloblastoma and implications for therapy. *Mol Cancer Ther.* **11,** 288–297 (2012).
33. Dudu, V., Able, R. A., Jr., Rotari, V., Kong, Q. & Vazquez, M. Role of Epidermal Growth Factor-Triggered PI3K/Akt Signaling in the Migration of Medulloblastoma-Derived Cells. *Cellular and molecular bioengineering.* **5,** 502–413 (2012).
34. Fogarty, M. P., Emmenegger, B. A., Grasfeder, L. L., Oliver, T. G. & Wechsler-Reya, R. J. Fibroblast growth factor blocks Sonic hedgehog signaling in neuronal precursors and tumor cells. *Proc Natl Acad Sci USA* **104,** 2973–2978 (2007).
35. Emmenegger, B. A. *et al.* Distinct roles for fibroblast growth factor signaling in cerebellar development and medulloblastoma. *Oncogene.* **32,** 4181–4188 (2013).
36. Baumgartner, M. *et al.* The Nck-interacting kinase phosphorylates ERM proteins for formation of lamellipodium by growth factors. *Proc Natl Acad Sci USA* **103,** 13391–13396 (2006).
37. Ma, M. & Baumgartner, M. Intracellular Theileria annulata promote invasive cell motility through kinase regulation of the host actin cytoskeleton. *PLoS Pathog.* **10,** e1004003, doi: 10.1371/journal.ppat.1004003 (2014).
38. Keles, G. E. *et al.* Establishment and characterization of four human medulloblastoma-derived cell lines. *Oncology research.* **7,** 493–503 (1995).
39. Fiaschetti, G. *et al.* NOTCH ligands JAG1 and JAG2 as critical pro-survival factors in childhood medulloblastoma. *Acta Neuropathol Commun.* **2,** 39, doi: 10.1186/2051-5960-2-39 (2014).
40. Ellison, D. W. *et al.* Medulloblastoma: clinicopathological correlates of SHH, WNT, and non-SHH/WNT molecular subgroups. *Acta neuropathologica.* **121,** 381–396 (2011).

Acknowledgements

The authors thank Tamara Hüsser for technical assistance with primary tumour cell line establishment and Tarek Shalaby for critical comments and suggestions. This study was supported by grants from the Child and Cancer Foundation to M.A.G., and from the Swiss National Science Foundation to G.S. (SNF_205321-134783) and to M.B. (SNF_31004A-144090/1).

Author Contributions

K.S.K. performed the experiments and contributed to designing the study, preparing the figures and writing the manuscript. M.P. and J.K. designed and wrote the software tools and prepared figure 2, I.B. and M.W. provided primary cell material and critically reviewed the manuscript, G.S. supervised software design and implementation and reviewed manuscript, M.G. helped drafting the study and critically reviewed the manuscript, M.B. designed study and wrote manuscript.

Additional Information

Supplementary information accompanies this paper at http://www.nature.com/srep

Competing financial interests: The authors declare no competing financial interests.

How to cite this article: Kumar, K. S. *et al.* Computer-assisted quantification of motile and invasive capabilities of cancer cells. *Sci. Rep.* **5**, 15338; doi: 10.1038/srep15338 (2015).

Manuscript 2: Supplementary Information

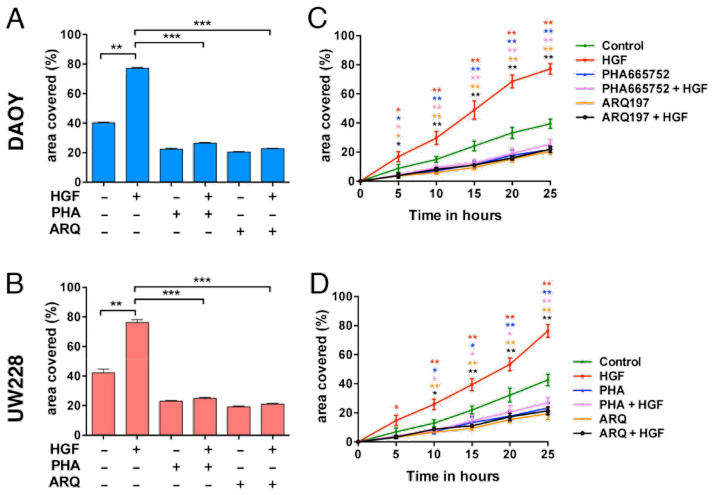


Fig. S1: aZEcs, aMDIcs and aSDIcs quantification reliably confirm manul measurements of HGF-induced cell dissemination under serum-free conditions. A, B) Means and SDs of % area covered from three independent zone infiltration experiments using aZEcs in DAOY (A) or UW228 (B) at T_{24h} after stimulation with 20 ng/ml HGF or treatment with c-Met inhibitors PHA665752 or ARQ197 (125 nM each) or both. **C, D)** Time-lapsed mean and SD quantifications of % area covered from three independent zone infiltration experiments using aZEcs in DAOY (C) or UW228 (D) after stimulation with 20 ng/ml HGF or treatment with c-Met inhibitors PHA665752 and ARQ197 (125 nM each) or both.

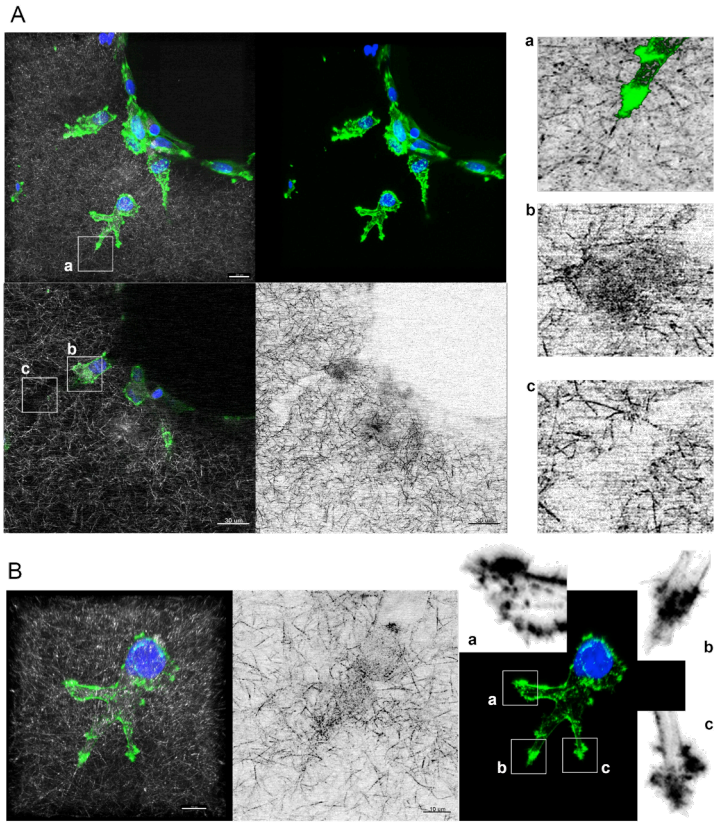


Fig. S2 Dissemination of DAOY cells from microbeads into collagen. 3D surpass images of LA-EGFP-expressing DAOY cells migrating of microbeads into collagen gel. a, b and c are 4x magnifications of the boxed areas and show invasion-dependent modifications in the collagen gel. Green: LA-EGFP, blue: hoechst DNA staining, black and white: reflection image of collagen fibres. B) Higher magnification image of single invading cell.

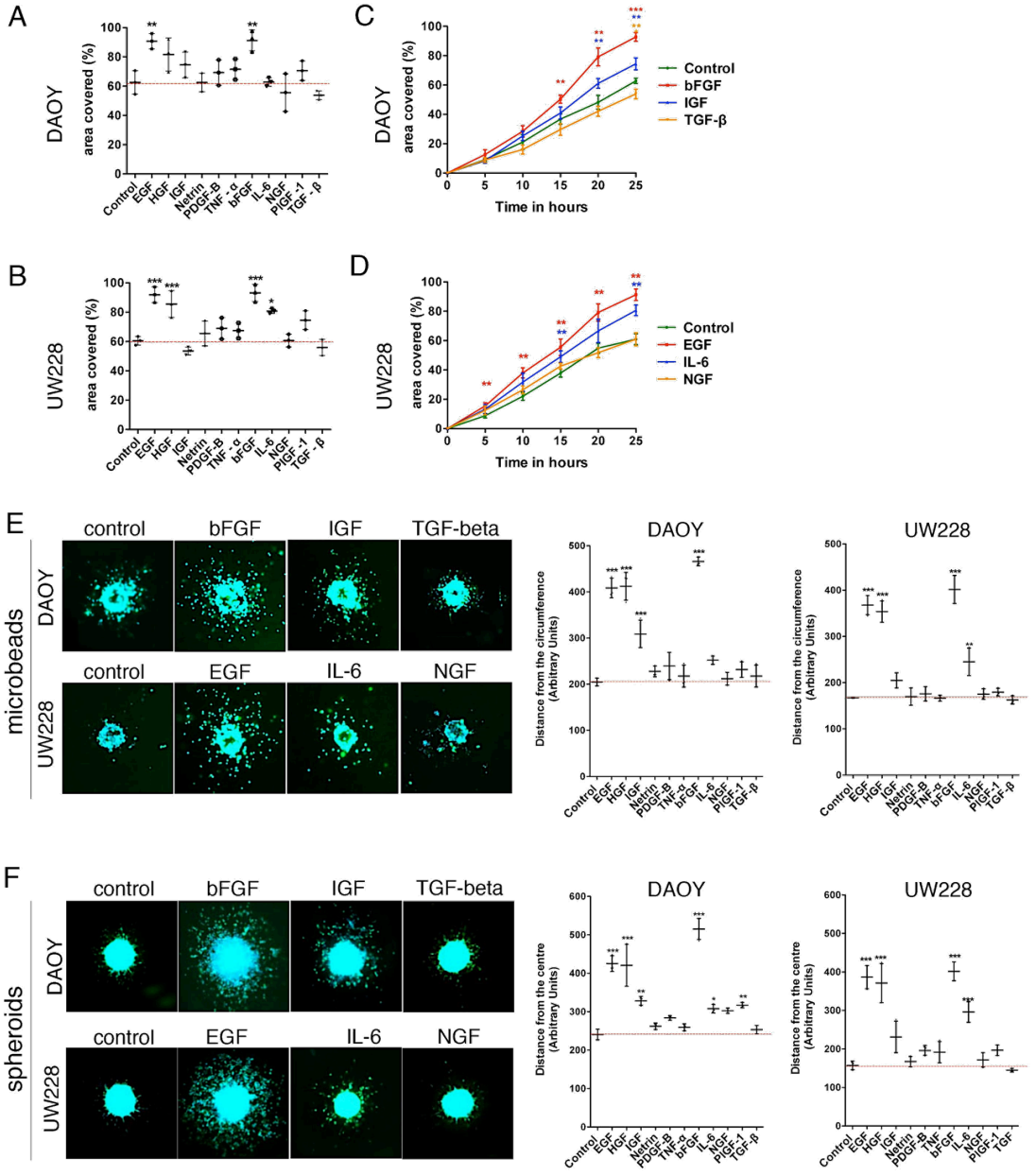


Fig. S3 Selective induction of cell dissemination with growth factors under serum-free conditions. A, B) End-point quantification of % area covered in zone infiltration assay using aZEcs. Means and SDs of three independent experiments with DAOY (A) or UW228 (B) cells at T_{24h} after stimulation with factors as indicated are shown. Concentrations of growth factors/cytokines as in 4A,B. **C, D)** Time-lapsed mean and SD quantifications of % area covered from three independent zone infiltration experiments using aZEcs in DAOY (C) or UW228 (D) cells stimulated with factors as indicated. **E)** Representative images of 100x magnified microbeads coated with DAOY or UW228 cells after 24 h -/+ stimulation with bFGF, IGF or TGF-beta (DAOY) or EGF, IL-6 or TGF-beta (UW228). LA-EGFP in green, Hoechst staining in blue. Right panels: quantification of means and SDs of cell dissemination/invasion from three independent experiments using aMDIcs. **F)** Representative images of 50x magnified spheroids of DAOY or UW228 cells after 24 h -/+ stimulation with bFGF, IGF or TGF-beta (DAOY) or EGF, IL-6 or NGF (UW228); LA-EGFP in green, Hoechst staining in blue. Right panels: Quantification of distance of cell dissemination/invasion using aSDIcs.

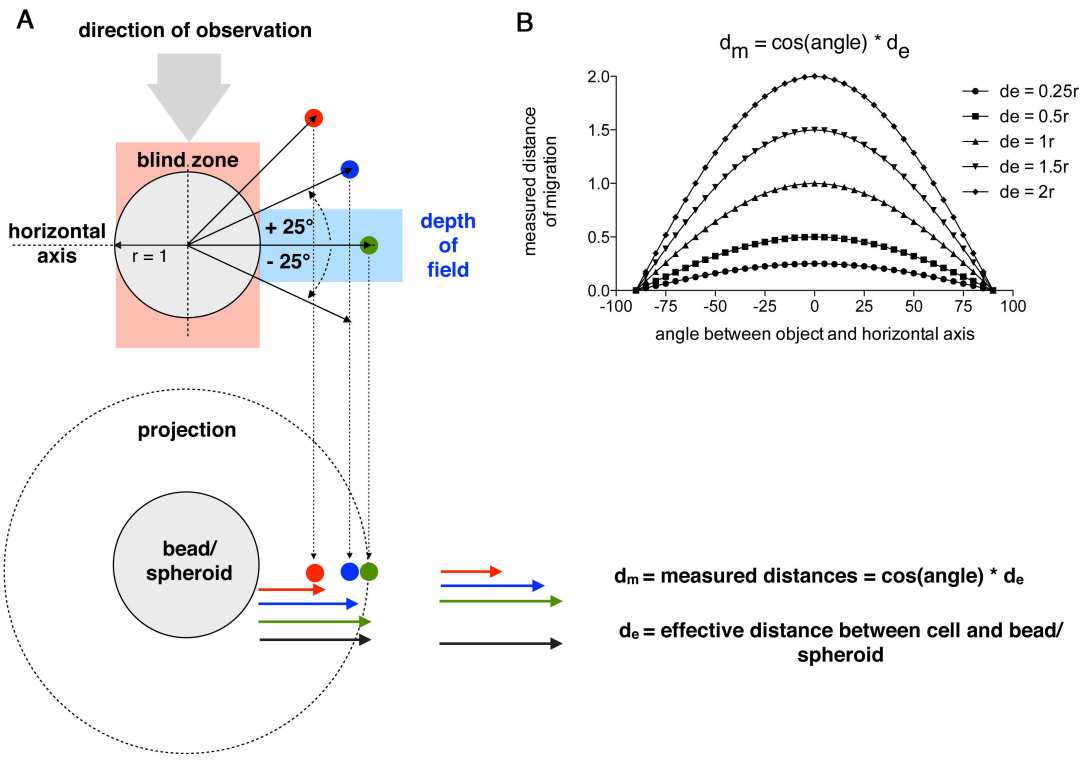


Fig. S4 Schematic overview of angle-distance relationship for distance quantification. (A) The images used for the aMDIcs and aSDIcs quantification are projections of 3D spaces acquired by non-confocal microscopy with a depth of field of approximately 50 µm (blue highlight). The area above and below the beads is not visible (red highlight). The projection of objects (cells) from the 3D space onto a 2D images causes the underestimation of distances according to the formula d_m = cos(angle) * d_e (d_m: measured distance, d_e: effective distance of cell from bead surface). **(B)** XY plot shows relationship between angle of observation and the deviation of the measured distance from the effective distance. Measured distances of objects located within 25° above and below the horizontal axis deviate from the effective distance between object and beads surface by less than 10%.

Supplementary Video 1

Collagen embedded microbead coated with DAOY cells: Inverted grey scale of EGFP fluorescence of DAOY cells expressing LA-EGFP. Cells were seeded on microbeads and embedded in collagen. 20 x objective, recording time 18 h, 30 min intervals, 10 frames/second.

Supplementary Video 2

Collagen embedded microbead coated with DAOY cells and stimulated with HGF: Inverted grey scale of EGFP fluorescence of DAOY cells expressing LA-EGFP. Cells were seeded on microbeads. embedded in collagen and stimulated with 20 ng/ml HGF. 20 x objective, recording time 18 h, 30 min intervals, 10 frames/second.

Supplementary Video 3

Collagen embedded microbead coated with UW228 cells: Inverted grey scale of EGFP fluorescence of UW228 cells expressing LA-EGFP. Cells were seeded on microbeads and

embedded in collagen. 20 x objective, recording time 18 h, 30 min intervals, 10 frames/second.

Supplementary Video 4

Collagen embedded microbead coated with UW228 cells and stimulated with HGF: Inverted grey scale of EGFP fluorescence of UW228 cells expressing LA-EGFP. Cells were seeded on microbeads and embedded in collagen and stimulated with 20 ng/ml HGF. 20 x objective, recording time 18 h, 30 min intervals, 10 frames/second.

Supplementary Video 5

Collagen embedded DAOY spheroid: Inverted grey scale of EGFP fluorescence of DAOY cells expressing LA-EGFP. Cells were grown to spheroids and embedded in collagen. 20 x objective, recording time 18 h, 30 min intervals, 10 frames/second.

Supplementary Video 6

Collagen embedded DAOY spheroid stimulated with EGF: Inverted grey scale of EGFP fluorescence of DAOY cells expressing LA-EGFP. Cells were grown to spheroids, embedded in collagen and stimulated with 30 ng/ml EGF. 20 x objective, recording time 18 h, 30 min intervals, 10 frames/second.

4.4 Manuscript 3
(Prepared for submission)

Antagonizing crosstalk between bFGF and TGFβ signaling controls tissue infiltration in medulloblastoma

Karthiga Santhana Kumar[1], Anuja Neve[1], Ana Guerreiro[2], Claudia Fischer[2], Elisabeth J. Rushing[3], Michael D. Taylor[2], Dimitra Tripolitsioti[1], Lena Harder[4], Michael A. Grotzer[1,5] and Martin Baumgartner[1.]

Affiliations:

[1]Department of Oncology, Children's Research Center, University Children's Hospital Zürich, August-Forel Strasse 1, CH-8008 Zürich, Switzerland

[2]Department of Laboratory Medicine and Pathobiology, University of Toronto, Toronto, Ontario M5S1A1, Canada Program in Developmental and Stem Cell Biology, Hospital for Sick Children, Toronto, Ontario M5G 1X8, Canada Division of Neurosurgery, Hospital for Sick Children, Toronto, Ontario M5G 1X8, Canada.

[3]Institute of Neuropathology, University Hospital Zürich, Schmelzenbergstrasse 12, 8091 CH-Zürich. Switzerland

[4]Paediatric Leukemia group, Department of Oncology, Children's Research Center, University Children's Hospital Zürich, August-Forel Strasse 1, CH-8008 Zürich, Switzerland

[5]Department of Oncology, University Children's Hospital Zürich, Steinwiesstrasse 75, CH-8032 Zürich, Switzerland

Correspondence: Martin.Baumgartner@kispi.uzh.ch

Summary:

How medulloblastoma (MB), a metastatic pediatric tumor of the cerebellum, infiltrates the brain tissue is poorly understood.

We found that microenvironment-derived bFGF promotes tumor cell infiltration through the adaptor FRS2. FRS2 function is antagonized by TGF-β, which induces a contractile, non-motile cell phenotype through ROCK activation and FRS2 repression. Without TGF-β, bFGF-FRS2 function attenuates ROCK and enables cell motility through Rac-PAK and ERK1/2. At high concentrations of bFGF, TGF-β rescues motility impeded by a FGFR1-dependent negative feed-back and triggers brain tissue infiltration.

This antagonistic crosstalk between bFGF and TGF-β signaling pathways tunes motile and invasive properties of brain tumor cells for tissue infiltration under varying environmental conditions and identified FRS2 as a novel drug target.

Keywords:

Medulloblastoma, FGFR1, FRS2, bFGF, TGFβ, cell motility, microenvironment

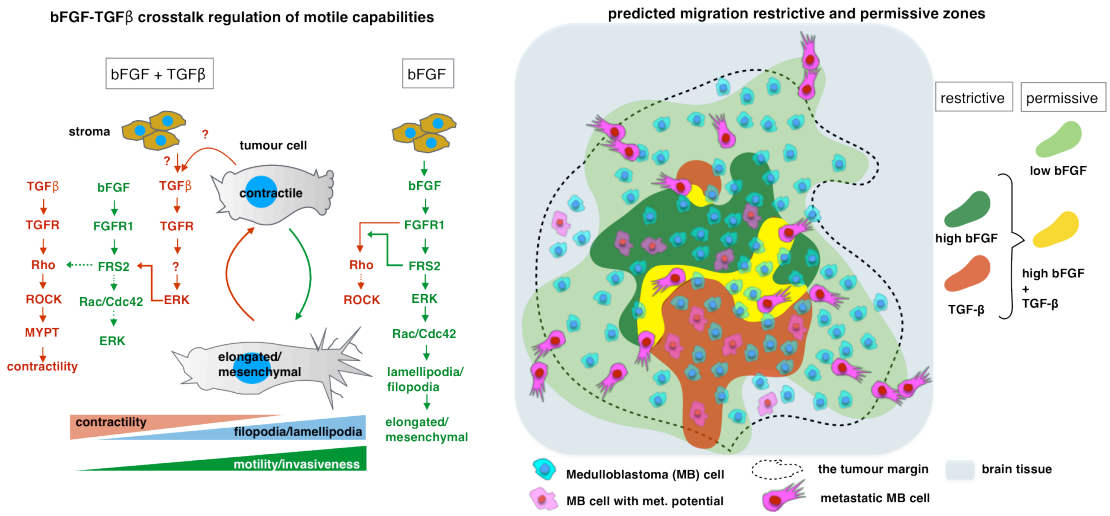
Significance:

Diffuse brain tissue infiltration in MB and other brain tumors poses a major challenge in the clinic and results in high mortality and morbidity. No targeted therapy is available and capable of reducing tissue infiltration. We report that soluble growth factors from the brain microenvironment control brain tissue infiltration by modulating the motile and invasive capabilities of the tumor cells. The underlying molecular mechanism involves an antagonistic crosstalk between bFGF and TGF-β signaling, which highlights a novel tumor cell mechanism to cope with the highly variable abundance of growth factors in the tumor microenvironment. We identified the FRS2 adaptor protein as a promising novel target and demonstrated that blocking its function prevented dissemination in MB.

Highlights:

- bFGF promotes brain tissue infiltration in medulloblastoma through the adaptor protein FRS2
- bFGF-driven FRS2 function is antagonized by TGF-β signaling
- TGF-β signaling elicits a contractile cell phenotype through Rho Kinase ROCK
- Blockade of microenvironment-induced FRS2 function prevents brain tissue infiltration

Graphical Abstract:



Introduction:

Medulloblastoma (MB) is the most prevalent malignant pediatric brain tumor and is a major cause of cancer-related death in children. MB originates from neural progenitor cells of the developing cerebellum (Hatten and Roussel, 2011; Marino, 2005) and is classified into four molecular subgroups WNT (wingless), SHH (sonic hedgehog), Group 3 (G3) and G4

(Northcott et al., 2012). MB cells display an inherent propensity to infiltrate the brain tissue and to disseminate along the leptomeninges (Wu et al., 2012). The dissemination process is a high risk factor, can cause incurable MB disease and necessities aggressive, non-specific regimens that often cause disabling side effects in long-term survivors (Bouffet et al., 1992; Fruhwald and Plass, 2002; Kiltie et al., 1997). Novel therapies targeting the dissemination process are thus urgently needed.

The molecular mechanisms that tune MB leptomeningeal dissemination remains enigmatic. Expression profiling (MacDonald et al., 2001) and clonal selection analyses of MB metastases (Wu et al., 2012) provided insights into the genetic control of MB metastasis. Metastasis-associated, differentially regulated genes are correlated with de-regulated kinase functions, abnormal actin cytoskeleton regulation and resistance to chemotherapy (MacDonald et al., 2001). Apart from cell intrinsic factors, MB tumor progression or regression depends on the tumor microenvironment (Lu et al., 2012; Wong and Rustgi, 2013). Growth factor (GF) combinations and concentration gradients in the tumor microenvironment define the extent of cancer cell dissemination and invasion (Bissell and Radisky, 2001; Wells et al., 2002). Few studies have assessed the simultaneous effects of two or more growth factors in different cancers (Shirakihara et al., 2011; Snoussi et al., 2010). No similar study has been carried out in MB.

We identified basic Fibroblast Growth Factor (bFGF) as a strong promoter of MB cell migration and invasion (Kumar et al., 2015). bFGF is produced in neurons and glial cells, and tunes diverse functions in the CNS, including developmental patterning, neurogenesis, axonal growth and neuroprotection (Gomez-Pinilla et al., 1994; Reuss and von Bohlen und Halbach, 2003). bFGF and its main tyrosine kinase receptors FGFR1 and FGFR4 control proliferation, survival, migration and differentiation (Holland and Varmus, 1998; Hutley et al., 2004; Murphy et al., 1990). Aberrations in FGFR and FGF function have been implicated in various human cancers (Cappellen et al., 1999; Freier et al., 2007; Jacquemier et al., 1994; Kunii et al., 2008; Turner and Grose, 2010). FGFRs signal via Fibroblast Receptor Substrate 2 (FRS2)-dependent (RAS/MAPK and PI3K/AKT) and FRS2-independent (PLC-γ) pathways (Turner and Grose, 2010). FRS2 directly associates with FGFRs via its phosphotyrosine binding domain (PTB) (Gotoh, 2008). Whereas our study suggested bFGF as a potent pro-migratory factor in MB (Kumar et al., 2015), another study found bFGF to inhibit SHH signaling and to block MB cell proliferation (Fogarty et al., 2007). Thus, context-dependent bFGF signaling may determine its contribution to MB pathogenesis. Transforming Growth Factor beta (TGF-β), which signals through its receptors (TβRI and TβRII), contributes to context-dependent cell functions. TGF-β can initiate either the canonical SMAD-dependent or the non-canonical SMAD-independent pathways (Derynck and Zhang, 2003; Massague, 2012) to regulate cell proliferation, wound healing, stemness and cell differentiation (Ashcroft et al., 1999; Heldin et al., 2009). Most cell types in the CNS act as a source of TGF-β and respond to TGF-β stimulation, which allows for developmental control of the CNS, neuronal transmission and neuroendocrine regulation (Dobolyi et al., 2012; Krieglstein et al., 2011). The context of TGF-β signaling dictates whether it either acts tumor-repressive or tumor-supportive in human cancers, where it can induce epithelial to mesenchymal transition (EMT), cell mobilization and eventually metastasis (Bierie and Moses, 2006;

Massague, 2008). In MB, positive nuclear staining of SMAD3 and canonical activation of TGF-β signaling antagonizes SHH pathway function and correlates with prolonged survival in MB (Aref et al., 2013). In contrast, TGF-β signaling induced in MB tumor-associated regulatory T cells prevents cytotoxic T-cell mediated killing of the tumor cells (Gate et al., 2014).
How the tumor microenvironment controls pro-metastatic capabilities in MB tumor cells is currently not known and strategies to block those are not available. Therefore, we investigated whether bFGF-induced cell migration and invasion in MB could be a central component of micro-environmental control of pro-metastatic MB tumor cell functions. Furthermore, we determined whether the blockade of key components of the FGFR signaling blocks those functions and whether additional pathways modulate FGFR signaling. Using laboratory and patient-derived xenograft MB tumor lines and *ex vivo* organotypic culture, we investigated the molecular mechanisms of growth factor control of MB brain tissue infiltration and explored the therapeutic potential of targeting FGFR signaling as anti-metastatic therapy.

Results:

bFGF promotes MB cell dissemination and its receptor is overexpressed in tumor tissue:
We previously identified bFGF as a strong promoter of cell dissemination using spheroid invasion assays (SIAs) and automated Cell Dissemination counter (aCDc) (Kumar et al., 2015). We confirmed the pro-migratory and pro-invasive effects of bFGF in established SHH MB cell lines (DAOY and UW228), MB patient derived xenografts of SHH MB (Med PDX1712), of G3 MB (Med PDX411, (Kumar et al., 2015)) and an atypical MB Med PDX311 (Figure 1A). No bFGF-induced migration and invasion was observed in the G3 MB line HD-MBO3 (Figure 1A). To determine which FGFRs are expressed in MB, we evaluated the expression levels of FGFR1 and FGFR4 at the protein and FGFR1-4, at the mRNA level. Immunohistochemical (IHC) data on an MB tissue microarray (TMA) showed that FGFR1 was expressed at moderate to high levels in 62% of the MB tumors (n = 129) as compared to 45% in the normal cerebellum tissue adjacent to the tumor (n = 11). 15% of the MB samples displayed high expression (Figure 1B). In addition, anti-FGFR1 IHC showed a strong positive staining for FGFR1 in G3 Med114FH and SHH Med1712 PDX (Figure 1C), while FGFR4 was not detectable in these samples (data not shown). Moreover, FGFR1 and FGFR4 mRNA expression was markedly increased in a small cohort of primary MB samples (Figure S1A). mRNA expression analysis indicated that FGFR1 was expressed predominantly in SHH subgroup MB cell lines (Figure 1D), whereas FGFR4 was expressed in group 3 lines. mRNA expression level analysis of bFGF using qRT-PCR demonstrated that bFGF was neither in MB cell lines nor in PDX cells highly expressed (Figure S1B). To determine the potential origin of bFGF *in vivo*, we performed anti-bFGF IHC analysis of a non-WNT/SHH MB tumor sample (Figure 1E), an MB TMA (Figure S1C), a G3 MB PDX (Figure S1D) as well as two additional MB validated clinical isolates (B2-54, C2-25) (Figure S1E). The analysis revealed a relatively weakly positive tumor tissue and the accumulation of strongly bFGF-positive cells in proximity of and within the tumor. This suggested that the source of bFGF was a cell infiltrating the tumor rather than the autocrine production by the tumor cells. Collectively, this signifies that FGFR1 is overexpressed in MB and bFGF present prevalently in MB tumor microenvironment.

bFGF signals through FGFR, FRS2 and ERK and induces mesenchymal motility *in vitro*:
Blockade of FGFR, ERK and PAK1 with their respective inhibitors (Figure S2A) reduced bFGF-induced dissemination in DAOY, UW228 and Med311 PDX cells to basal (FGFRi, ERKi) or intermediate levels (PAK1i) (Figure 2A). The effective concentration of BGJ398 was determined by titration (Figure S2B) and the inhibitors were used at concentrations not affecting cell viability (Figure S2C, 2D). Inhibitors for other putative effectors such as PKC, PI3-K or JNK did not prevent bFGF-induced collagen infiltration (Figure S2E) but blocked it when induced by EGF or HGF (Figure S2F). Depletion of ERK1 or ERK2 using siRNA also significantly reduced bFGF-induced migration and invasion (Figure 2B). siRNA depletion of ERK1/2 or pharmacological ERK inhibition did only moderately affect EGF-, and not affect HGF-induced dissemination in DAOY (Figure 2C), UW228 and Med311 cells (Figure S2G). Immunoblot (IB) analysis revealed increased phosphorylation of the FGFR1 adapter protein Fibroblast growth factor Receptor Substrate-2 (FRS2) and of ERK1/2 after bFGF stimulation (Figure 2D), indicating a bFGF-FGFR-FRS2-ERK signaling axis. Confocal imaging of bFGF-treated DAOY spheroids embedded in collagen revealed the formation of highly dynamic lamellipodia- and filopodia-like structures at the leading edge of invading cells (Figure 2E, Video S1). These protrusive structures are a characteristic feature of the mesenchymal mode of motility and hallmarks of invading cancer cells (Jacquemet et al., 2015). Impairing the function of the Arp 2/3 complex, which is necessary for the formation of lamellipodia and filopodia (Korobova and Svitkina, 2008; Suraneni et al., 2012) using CK666 (Hetrick et al., 2013), completely abrogated bFGF-induced dissemination (Figure S2H). We used the actin-based motor protein myosin X as a filopodia marker because it is known to assemble at the tip of filopodia in particular also during neuronal migration (Kerber and Cheney, 2011; Lai et al., 2015). DAOY LA-GFP cells stimulated with bFGF stained positive for myosin X at the tips of the filopodia-like structures both in collagen-coated 2D environment (Figure 2F) as well as when embedded in collagen in a 3D environment (Figure 2G). Importantly, filopodia-like invasion structures were also observed in bFGF-stimulated MB cells invading the cerebellar tissue *ex vivo* (Figure S2I). Thus, bFGF triggers a mesenchymal mode of motility in 3D that is dependent on branched and parallel bundled F-actin polymerization for lamellipodia and filopodia formation and tissue invasion.

TGF-β signaling antagonizes bFGF-induced cell migration:
To reveal additive, synergistic and/or antagonistic growth factor signaling, we carried out a combinatorial growth factor screen in 3D. We adapted Plackett Burman (PB) design based on two-level (High and Low) Hadamard screening matrices (Figure S3 and Table 2). Surprisingly, we detected no additive or synergetic effects among the combinations tested (Figure S4A). Combination 5, which contained high levels of bFGF did not promote significant cell migration (Figure S4A). This strongly indicated the presence of an antagonistic signaling pathway preventing bFGF-induced migration. Therefore, we performed SIAs with MB cells co-stimulated with bFGF plus one additional growth factor/cytokine present in high levels in combination 5. These individual combinations revealed that Transforming Growth Factor-β (TGF-β) abrogated cell migration induced by bFGF in DAOY, UW228 and Med 311 PDX cells (Figure 3A). In contrast, migration induced by EGF and HGF remained unaffected (Figure 3B). Blocking TGFR with its inhibitor LY2157299 rescued bFGF-induced migration in co-stimulated

cells in a dose dependent manner (Figures 3C, S4B), confirming the antagonizing effect of TGF-β on bFGF signaling. We titrated the minimal pro-migratory and the the minimal blocking concentrations of bFGF and TGF-β, respectively (Figure 3Db,c,d). Treating the MB cells with concentrations up to 200 ng/ml TGF-β did not increase the antagonistic effect, while concentrations below 20 ng/ml TGF-β did not effectively block bFGF-induced migration (Figure 3Db). TGF-β blockade of bFGF-induced migration and invasion is immediate and most likely independent of transcriptional control (Figure 3E left), as acute TGF-β was able to mitigate bFGF function even after 10 h pre-treatment with bFGF (Figure 3E right). Importantly, TGF-β co-stimulation with bFGF did not induce any cytotoxicity (data not shown). Invariably, bFGF-driven invading MB cells displayed myosin X-decorated filopodia protruding from the invasive lamellipodia at the leading edge (Figure 3F). Stimulation with TGF-β markedly reduced the number of filopodia and abrogated myosin X localization to filopodia tips (Figure 3F). This signifies that TGF-β confines bFGF signaling by affecting bFGF-induced mesenchymal motility. Together, the above data identify the TGF-β pathway as a novel regulator of FGFR1 signaling in MB cells by specifically restraining bFGF-induced mesenchymal motility.

TGF-β–ROCK–induced contractility impairs cell motility triggered by bFGF:

qRT-PCR analysis detected predominant expression of TGFRB1 and TGFRB2 in MB cells and a moderate level of TGF-β (Figure S5A), which was not sufficient to prevent bFGF-induced migration *in vitro*. The requirement of a minimal concentration of 20 ng/ml TGF-β for effective FGFR1 blockade indicated a resilience of bFGF signaling to TGF-β exposure. To understand the suspected threshold regulation of FGFR signaling, we investigated the downstream effectors in both pathways. Non-canonical TGFR signaling can induce a contractile phenotype through the Rho-associated kinase ROCK (Maddox and Burridge, 2003; Zhang, 2009). Exposing MB cells to TGF-β increased the phosphorylation of the ROCK substrate Myosin light chain phosphatase 1 (MYPT1), both in the absence and presence of bFGF (Figures 4A, S5B). Down-regulation of ROCK using siRNA or inhibition of ROCK using Y27632 increased migration and invasion and converted TGF-β into a pro-migratory factor (Figures 4B, S5C). We therefore blocked ROCK with either Y27632 or the unrelated ROCK inhibitor H1152 in cells stimulated with bFGF and TGF-β. Both inhibitors rescued migration and invasion in the presence of TGF-β, to levels comparable to bFGF alone (Figure S5D). This effect is specific for ROCK, as inhibition of ERK or PAK1 caused no rescue in the presence of TGF-β (Figure S5D). Moreover, down-regulation of either ROCK1 or ROCK2 with siRNA also blunted TGF-β blockade of bFGF-induced cell dissemination (Figure 4C). Since ROCK1/2 activation causes cell contraction (Maddox and Burridge, 2003), we examined if cellular contraction counters MB cell dissemination. We blocked myosin II with blebbistatin and found that this treatment completely rescued bFGF-induced migration and invasion in the presence of TGF-β (Figure 4D). Live confocal imaging of collagen-1 embedded, TGF-β-treated DAOY cells exhibited a contracted, rounded phenotype compared to un-stimulated or bFGF-treated cells (Figure 4E, Video S2), which displayed numerous lamellipodia- and filopodia-like structures. ROCK inhibition reverted the contractile phenotype and triggered massive filopodia formation in collagen (Figure 4E). Interestingly, inhibition of FGFR with BGJ398 (Guagnano et al., 2011) (Figure 4F) or down-regulation of FRS2 using siRNA (Figures 4G, S5E) increased phosphorylation of MYPT1, which was further increased in cells co-stimulated

with bFGF and TGF-β (Figures 4F, 4G, S5E). This suggested that FGFR1-FRS2 signaling restricted ROCK activation in the absence of TGF-β (Figure 5E). Furthermore, the specific increase in phosphorylation of MYPT1 in cells co-stimulated with bFGF and TGF-β depicts that rapid induction of ROCK by TGF-β overrides the inhibitory effect of FRS2 on ROCK activation, resulting in the net increase in contractility. If true, it should be reflected in a differential activation of the Rho family GTPases Ras homology member A (RhoA) and Ras-related C3 botulinum substrate 1 (Rac 1), which are activated downstream of TGF-β and bFGF signaling, respectively. Indeed, we found RhoA activity increased when cells were stimulated with TGF-β while Rac activity remained unaltered (Figure 4H). Conversely, bFGF stimulation caused increased Rac activity but did not affect RhoA. Co-stimulation with bFGF and TGF-β completely abrogated bFGF-induced Rac activation, suggesting that TGF-β also interferes with FGFR signaling upstream of Rac activation (Figure 4H). Deterring bFGF signaling by targeting either FRS2 using siRNA or by blocking FGFR signaling using BGJ398, led to a general increase in RhoA in stimulated cells (Figure 4H), which emphasizes the inhibitory effect of FRS2 on ROCK activity. The activity of Cell division cycle 42 (Cdc42) remained unchanged in non-treated and growth factor-treated cells.

Collectively these findings demonstrate that ROCK activation by TGF-β represses the formation of filopodia-like structures in MB cells and antagonizes mesenchymal cell migration. It furthermore identified a morphological switch that is regulated by the reciprocal regulation of RhoA and Rac by TGF-β and bFGF signaling, which ultimately determines the migratory and invasive potential of MB cells (Figure 5E).

TGF-β inactivates FRS2 and attenuates bFGF-dependent dissemination:

ERK1/2 can regulate FRS2 function by decreasing its tyrosine phosphorylation (Zhou et al., 2009). Both bFGF and TGF-β activate ERK1/2 (Figure 5A), implying that FRS2 could be negatively regulated both via a negative feedback loop as well as through TGF-β-induced ERK1/2 activation. Co-stimulation with bFGF and TGF-β significantly reduced tyrosine phosphorylation of FRS2 (Figure 5B, 5C), indicating that TGF-β obstructs activation of FRS2. In cells co-stimulated with bFGF and TGF-β, inhibition of ERK with SCH772984 rescued the phosphorylation of FRS2 (Figure 5B, 5C). In contrast, inhibition of ERK1/2 in bFGF-only stimulated cells did not cause an increase in FRS2 phosphorylation. Thus, reduced FRS2 phosphorylation in co-stimulated cells is rather due to TGF-β-induced ERK activity and not due to a negative feedback. FRS2 binds to the juxta-membrane domain of FGFR1. Therefore, cortex morphology and dynamics might influence the proper positioning and activation of FRS2. As TGF-β triggers cortical contraction, we tested if contractility is associated with TGF-β inhibition of FRS2. Indeed, addition of blebbistatin to co-stimulated cells, which reverted TGF-β blockade of cell migration (Figure 4D), also rescued the phosphorylation of FRS2 (Figure 5D). Taken together, co-incidence of activated ERK1/2 and contractility is required for the effective mitigation of FRS2 activity, which occurs simultaneously in MB cells stimulated with TGF-β (Figure 5E).

Relative levels of bFGF and TGF-β determine motile and invasive capabilities:

To determine if the antagonistic crosstalk between bFGF and TGF-β signaling could take place in brain tissue as well, we studied the effects of bFGF on tumor cell dissemination in *ex*

vivo organotypic cerebellar slice culture (OCSC). In OCSCs, MB cells disseminated from implanted spheroids in the absence and under conditions of low (12.5 ng/ml) bFGF. Surprisingly, however, at 100 ng/ml of exogenous bFGF, dissemination of DAOY cells was reduced (Figure 6A). Immunofluorescence analysis on the cerebellar slices showed a strong signal for bFGF (Figure S6A) and bFGF was highly enriched around the tumor spheroid in OCSCs and accumulated at or near the tumor cell surface (Figure S6B). Thus, the inherent high concentration of bFGF in the cerebellum and addition of exogenous bFGF might increase a net concentration of bFGF in the cerebellar co-culture system above an inhibitory threshold. To test this possibility, we titrated bFGF to determine the maximal concentration that is still migration permissive. We found that migration diminished at concentrations above 100 ng/ml of bFGF *in vitro* (Figures 6B, S7A). TGF-β prevented MB cell dissemination *ex vivo in the* absence of exogenous bFGF (Figure 6A) and blocked bFGF-induced dissemination up to 100 ng/ml *in vitro* (Figure 6B). However, in the presence of very high concentrations of bFGF (10 µg/ml bFGF *in vitro* and 100 ng/ml bFGF *ex vivo*), TGF-β partially rescued migration and invasion (Figures 6A, 6B, S7B). Blocking TGFR catalytic activity with LY215729 deterred TGF-β restoration of bFGF-induced dissemination at very high concentrations of bFGF (Figure 6B). Likewise, bFGF-induced dissemination was prevented at all concentrations, when the cells were treated with BGJ398 (Figure 6B). In order to mimic the *ex vivo* situation of constant exposure to bFGF and TGF-β for 5 days *in vitro,* and to exclude the possibility of TGF-β-induced cell death, we pre-treated the MB cells with bFGF without or with TGF-β and then performed a WST-1 assay and a SIA after a total of five days (Figure 6C). Very high concentration of bFGF did reduce the viability of DAOY cells after 48 h, whereas TGF-β did not influence the viability (Figure S8A, 8B). Reduced dissemination at very high concentration of bFGF in DAOY cells might thus be partially due to cell death caused by very high concentration of bFGF. The viability of Med311PDX remained unaltered at all concentrations of bFGF and TGF-β (Figure S8C, 8D).
Thus, MB cell viability is decreased under very high concentration of bFGF, and their migratory and invasion capability depends on the relative levels of bFGF and TGF-β in the microenvironment.

TGF-β impairs negative-feedback regulation of FRS2:
bFGF caused the phosphorylation of Y436 of FRS2 and the activation of downstream ERK1 and ERK2 in the absence of a detectable increase of MYPT1 phosphorylation (Figure 7A). Phosphorylation of Protein Kinase C (PKC) remained also largely unaltered independent of the concentration of bFGF. Depletion of FRS2 or treatment with BGJ398 prevented ERK1/2 activation while it increased the phosphorylation of MYPT1, indicating FRS2 repression of ROCK (Figures 7A, 4F, 4G). Stimulation of control cells with 10 µg/ml bFGF abrogated ERK1/2 activation, indicating that a negative feedback prevents excessive MAPK signaling (Figure 7A). Co-stimulation of sgFRS2 cells with 100 ng/ml bFGF + TGF-β increased, while co-stimulation with 10 µg/ml bFGF + TGF-β lowered the phosphorylation of MYPT1, indicating that TGF-β signaling is no longer dominant over bFGF signaling at very high concentration of bFGF (Figures 7A, S9). High concentrations of bFGF did neither increase RhoA and ROCK activity nor decrease Rac1 activity (Figure 7B). However, Rac1 activity remained high in bFGF (10 µg/ml)-treated cells co-stimulated with TGF-β (Figure 7B), suggesting a shift towards mesenchymal motility. No perceptible change in the activity of Cdc42 was observed (Figure

7B). Total phosphorylation of FRS2 was reduced in DAOY cells after 24 hours of treatment with very high concentrations of bFGF (Figure 7C). This reduction in FRS2 phosphorylation was reverted when ERK1/2 was inhibited with SCH772984 or when cells were co-stimulated TGF-β (Figure 7C). Thus, at very high concentrations of bFGF, FGFR signaling is self-inhibited by ERK1/2 through a negative feedback. This negative feedback is dampened in the presence of TGF-β, which maintains FRS2 in the active state to repress ROCK, and to promote MB cell dissemination (Figure S9).

FRS2: A potential anti-metastatic therapy target in medulloblastoma:
mRNA expression analysis of FRS2 revealed FRS2 over-expressed in MB compared to normal cerebellum (Figure 8A). FRS2 is expressed at varying levels in all sub-groups of established MB and Med PDX cell lines tested (Figure 8B). Like FRS2, FGFR-1 is also expressed to similar extents in all MB and Med PDX cell lines tested (Figure 1C). FRS2 depletion using siRNA completely abrogated bFGF-induced dissemination in DAOY, UW228 and Med311PDX cells (Figure 8C, 8D, S10A). In contrast, down regulation of FRS2 did not affect EGF or HGF-induced cell dissemination, confirming that FRS2 is an exclusive downstream effector of bFGF-induced dissemination (Figure 8D). Depletion of FRS2 completely ceased the potent pro-migratory bFGF signaling and activated contractility (Figure 7A), thus mimicking the TGF-β anti-migratory phenotype. This makes FRS2 an attractive anti-dissemination therapy target in MB, as targeting FRS2 will prevent dissemination in 2 ways: a) by inactivating bFGF signaling and b) by increasing contractility. Complete knockdown of FRS2 using CRISPR/Cas9 system (Figure S10B) potently blocked bFGF-induced MB cell dissemination *in vitro* (Figure 8E) and *ex vivo* (Figure 8G), while EGF and HGF induced dissemination remained unaffected (Figure 8E). Indirect inhibition of FRS2 using BGJ398, blocked bFGF induced dissemination in OCSC (Figure 8F), indicating that FGFR inhibition could be a potential anti-metastatic therapy for MB.

Discussion:

We identified bFGF as a central component of micro-environmental control of pro-metastatic tumor cell functions in MB. We found that bFGF expressing cells infiltrate the primary tumors in patient samples and in mouse xenografts, and using organotypic cell culture we show that endogenous bFGF triggers brain tissue infiltration of single tumor cells and tumor dissemination. bFGF-dependent migration and invasion is mediated by the FGFR adaptor protein FRS2 and pharmacological blockade of FGFR or CRISPR/Cas9-mediated knock-down of FRS2 prevent brain tissue infiltration. We discovered that TGF-β signaling tune pro-metastatic functions of FRS2 by modulating its activation and by causing a morphological switch to a migration-incompetent, contractile phenotype through Rho-ROCK activation. Conversely, bFGF signaling causes Rac activation and represses Rho-ROCK to trigger mesenchymal motility. In the context of low bFGF concentration, TGF-β completely abrogates migration and invasion. In the context of high bFGF, which normally causes auto-inhibition of FGFR signaling, TGF-β rescues migration and invasion by preventing negative feedback regulation. This antagonistic crosstalk between bFGF and TGF-β signaling highlights a novel tumor cell mechanism to cope with the highly variable abundance of growth factors

in the tumor microenvironment and identified FRS2 as a promising novel target to restrict metastatic dissemination in MB.

Using a 3D collagen invasion assay and automated image quantification, we identified bFGF-FGFR as a potent pro-migratory signaling pathway in MB. It promotes mesenchymal motility (Friedl and Wolf, 2009) via FRS2, PAK-1 and ERK1/2. This bFGF-induced mesenchymal motility in the tumor cells is countered by an inhibitory circuitry induced by TGF-β and involving the activation of RhoA-ROCK and the repression of FRS2 tyrosine phosphorylation. ROCK activation in mesenchymal cells causes cortical contractions and cell rounding (Nobes and Hall, 1999). Tumor cells exploit this cellular plasticity and adopt different motility modes during tissue invasion (Sahai and Marshall, 2003; Torka et al., 2006). Indeed, the antagonistic activity of FRS2 we observed towards the activation of ROCK suggests that FGFR1-FRS2 signaling stabilizes reprogramming of the cytoskeleton towards mesenchymal migration in the absence of TGF-β. However, in the presence of TGF-β, robust ROCK activation dominates the subtle repression by FRS2 and consequently TGF-β signaling blunts FGFR signaling. Consequently, ROCK repression promotes motility and invasiveness in MB, which was also observed in various other cancers (Adachi et al., 2011, Wei, 2016 #167; Yang and Kim, 2014). In contrast, the rounded Rho-ROCK-dependent invasion mode was for example identified to hallmark alveolar Rhabdomyosarcoma, the aggressive, metastatic variant of Rhabdomyosarcoma (Thuault et al., 2016).

The migration-enabled phenotype in bFGF-stimulated MB cells is characterized by high Rac1 and low RhoA activity and the formation of lamellipodia with myosinX-decorated filopodia. The function and regulation of Rho-family GTPases in neuronal cells is complex (Gonzalez-Billault et al., 2012) and in addition to the paradigmatic Rac1 and RhoA GTPases, additional family members are likely involved in balancing the cytoskeleton dynamics during MB invasion. Although we found that ROCK activity represses filopodia formation, its significance remains to be determined. Filopodia were proposed to drive cancer cell invasion, possibly by facilitating environment sensing and substrate tethering (Jacquemet et al., 2015). Filopodia play an important role during neuronal differentiation (da Silva and Dotti, 2002) and neuritogenesis (Dent et al., 2007), suggesting that filopodia at the leading edge of invading MB cells may act as sensors and provide directional and haptotactic cues essential for brain tissue infiltration.

Another mechanism of repressing bFGF-induced motility by TGF-β involves the inhibition of the activating tyrosine phosphorylation on FRS2. We show that this inhibition is mediated by a TGF-β-induced, ERK-dependent inactivation of FRS2. It may involve ERK activation by FRS2-independent mechanisms via EGF, PDGF or insulin signaling, which can phosphorylate threonine residues on FRS2α and contribute to the negative regulation of FGF signaling (Gotoh, 2008; Zhou et al., 2009). Here we show for the first time that TGF-β-induced ERK can also partake in the inhibition of FRS2 for pathological cell mobilization.

Growth factors and cytokines create gradients within the tumors and the surrounding stroma, resulting in graded exposure of the cancer cells to these factors (Thoma et al., 2014). bFGF-positive cells within the MB tumor mass indicated bFGF gradients within the

MB tumor microenvironment. These concentration gradients may become more complex when another signaling pathway is activated within the gradient zone as for example in the case of reciprocal TGF-β and WNT signaling (Guo and Wang, 2009). Our findings revealed that activated TGF-β signaling can determine the migratory outcome of MB cells inside a bFGF gradient, suggesting a contextual functionality of TGF-β signaling (Massague, 2012). Indeed, in regions of the tumor where the concentration of bFGF is very high, activated TGF-β provides a permissive migratory environment for MB cells, which is normally hindered by negative feedback regulation of MAPK pathway downstream of FGFR. In regions of low concentrations of bFGF, combined exposure to TGF-β is restrictive. Thus, TGF-β provides a migratory environment within the tumor regions with bFGF overabundance and guide responsive tumor cells towards the periphery, where TGF-β may cede to be anti-migratory due to concentrations lower than the threshold required to inhibit bFGF signaling. This is exemplified in our findings where bFGF is pro-migratory even at a concentration of 5 ng/ml while at least 20 ng/ml TGF-β are required for an effective anti-migratory effect. Additional parameters such as sulfation patterns and length of heparin sulphate chains, which is a potent cofactor of canonical FGF signaling, will determine the net outcome of GF gradient function (Matsuo and Kimura-Yoshida, 2013). A recent study found that bFGF blockade of SHH activation prevents MB outgrowth and suggested activators of FGF signaling as potentially useful for targeting SHH MB (Emmenegger et al., 2013). Our data call for caution in that regard as the effective bFGF concentrations and the antagonistic TGF-β crosstalk described herein matter and could trigger increased dissemination of the tumor. Independent of bFGF concentration and absence or presence of TGF-β, the sole determining factor of whether MB cells migrate and invade in response to bFGF is FRS2. Thus, FRS2 acts as a molecular hub for pro-invasive FGF signaling in MB, which renders it an attractive target for an anti-dissemination therapy approach.

We show that inhibition of FRS2 prevents dissemination of MB cells in 2 ways: a) by inactivating FGF signaling and b) by increasing contractility, making it a unique target for MB. However, there are no commercially available inhibitors for FRS2. Therefore, we have validated BGJ398, a potent FGFR1-3 selective small molecule receptor tyrosine kinase inhibitor (RTKi) as an indirect strategy to inactivate FRS2. FGFRs are mutation-free in MB (Vogelstein et al., 2013); hence it could be an attractive target. However, the use of the RTKi in the treatment of cancer is associated with the development of resistance (Corcoran and O'Driscoll, 2015) and FGF signaling plays a variety of important roles in the developing brain (Reuss and von Bohlen und Halbach, 2003). Therefore, as a means to spare the other functions of FGF signaling in the developing brain of the pediatric patients and to specifically halt MB cell dissemination, we propose to target FRS2. Towards that end, novel FRS2 inhibitors will be needed, which specifically disrupt the interactions between the PTB domain of FRS2 and FGFR1.

Author Contributions:

K.S.K. planned and conducted experiments and contributed to designing the study, preparing the figures and writing the manuscript, A.N., A.G., C.F. and D.T. planned and

conducted experiments, L.H. provided cell sorting expertise, E.R. analyzed IHC data, M.T. critically reviewed the manuscript, M.A.G. helped drafting the study and critically reviewed the manuscript, M.B. planned and conducted experiments, designed study and wrote manuscript.

Acknowledgments:

The authors thank John Silber and Till Milde for generously providing cell lines and Scott McComb and the Paediatric Leukaemia group for providing the lenti CRISPR constructs. This study was supported by grants from the Swiss National Science Foundation (SNF_31004A-144090/1), from the Werner and Hedy Berger-Janser Foundation and from the Swiss Cancer League to M.B. and from Childhood Cancer Switzerland to M.A.G. the authors have no conflicts of interest to declare.

Manuscript 3: Figures

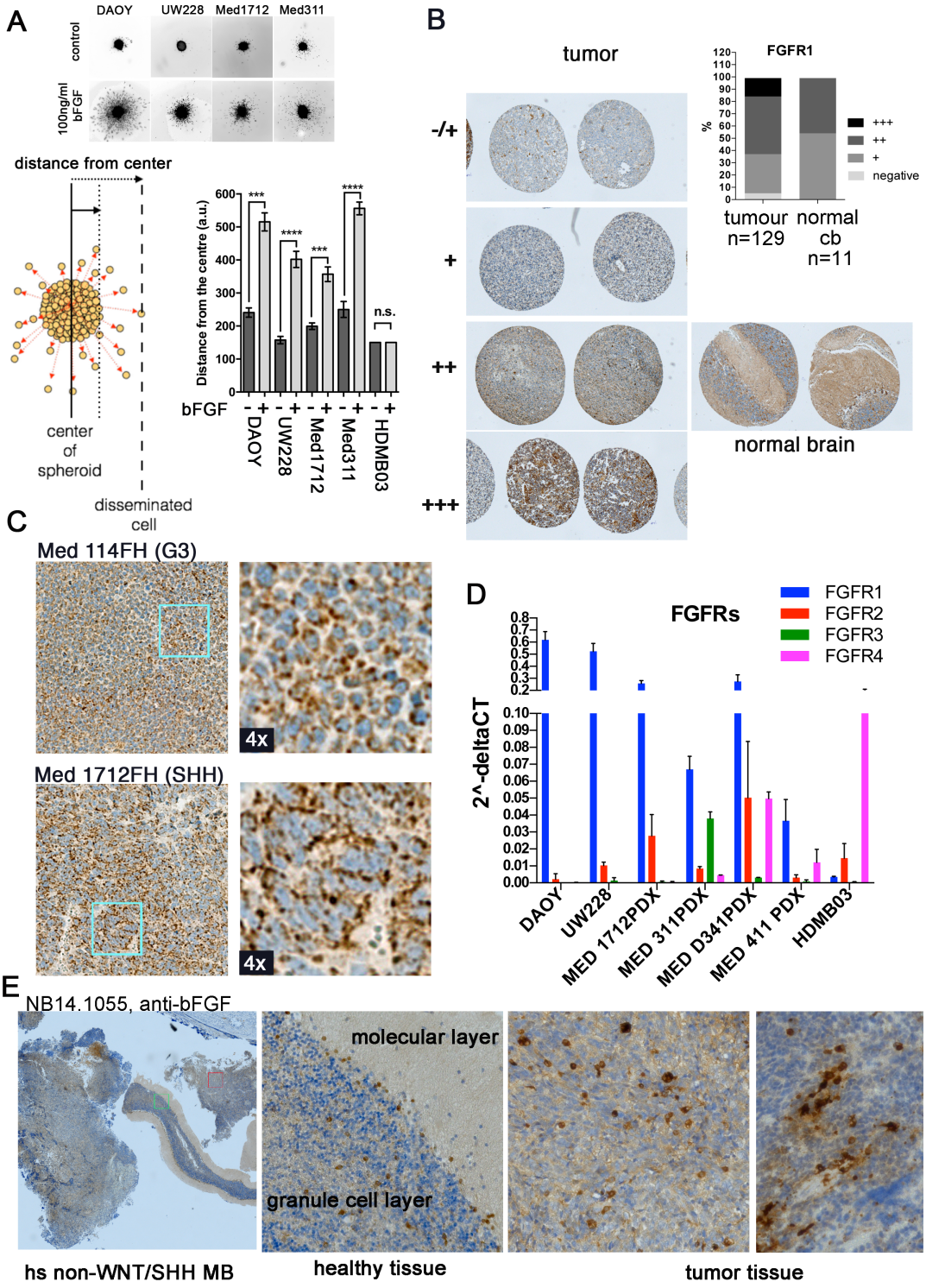


Figure 1: Up-regulation of functional FGFR1 signaling in MB (A) SIA with MB cell lines. Rep. images of spheroids after 24 h −/+ bFGF. Principle of quantification of distance of dissemination using aCDc. Quantification (means ± SD) of distance from centers of spheroids using aSDIcs from 3 independent experiments. **(B)** Rep. images of low, moderate and high expression of FGFR1 in MB TMA. Quantification of expression of FGFR1 in MB TMA using H-scoring. **(C)** IHC analysis of FGFR1 expression in xenografts of G3 (Med 114FH) and SHH (Med 1712FH) MB. **(D)** Comparative qRT-PCR expression analysis of FGFR1, FGFR2, FGFR3 and FGFR4 in established MB cell lines. **(E)** IHC analysis of bFGF expression in human non WNT/SHH MB sample. 4x magnif. of boxed areas from healthy and tumor tissue are shown.

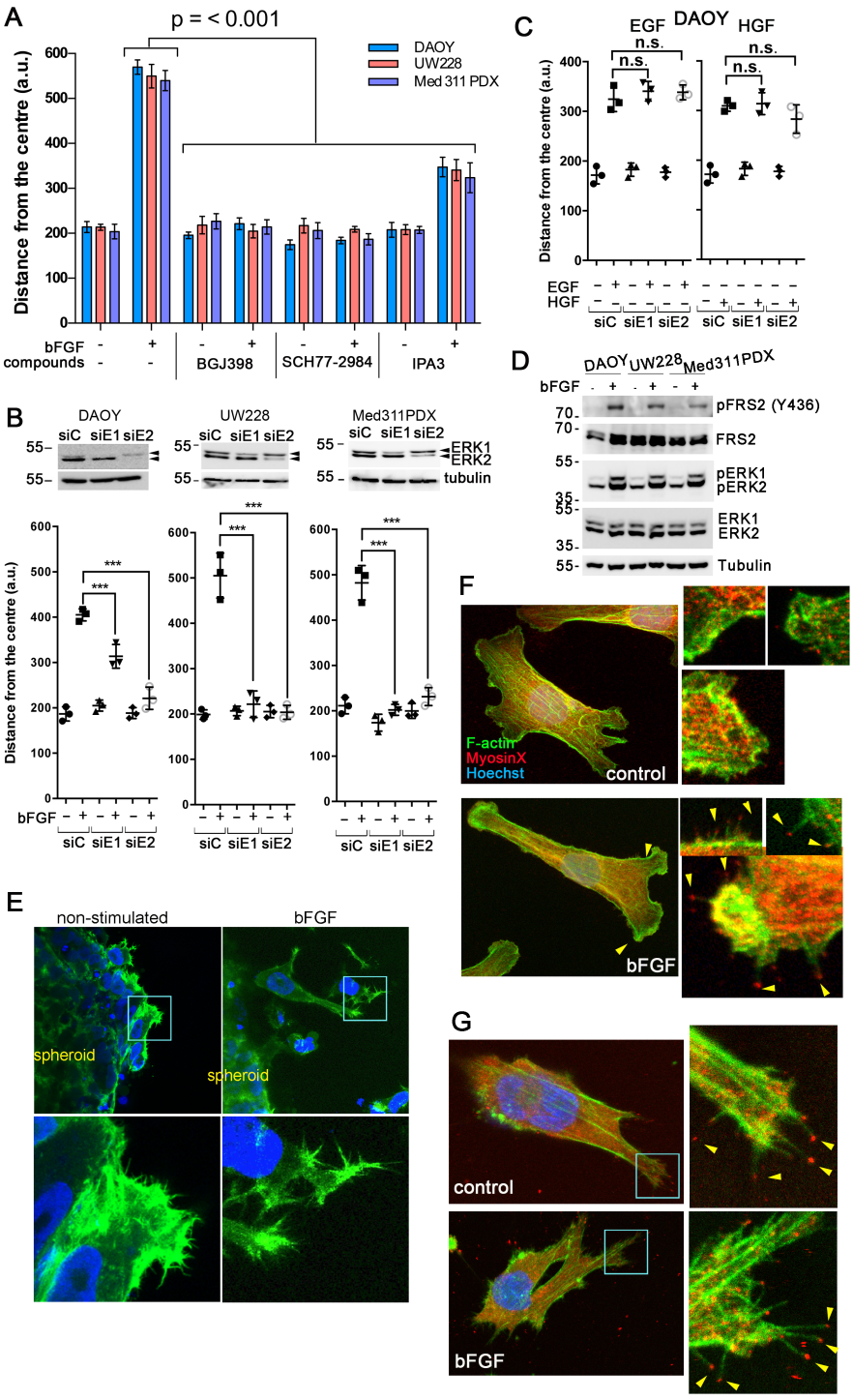


Figure 2: bFGF promotes ERK-dependent mesenchymal motility in MB cells. (A) Quantification (n=3, means ± SD) of SIA after 24h -/+ treatment with bFGF and/or BGJ398, SCH772984, IPA3. **(B)** IB of DAOY, UW228 and Med311PDX cell lysates post siMAPK3 (ERK1) or siMAPK1 (ERK2) transfection. Quantification (means ± SD) of SIA of DAOY, UW228 and Med311PDX cells transfected with siMAPK3 or siMAPK1 -/+ bFGF stimulation for 24 h. **(C)** same as in (B) expect only DAOY cells were used with -/+ HGF or EGF for 24 h. **(D)** IB of DAOY, UW228 and Med311PDX cell lysates after stimulation with bFGF for 10 minutes. Antibodies used as indicated to the right of each panel. **(E)** High resolution z-stack of SIA with DAOY cells -/+ bFGF stimulation for 24 h. Magnifications are 4x of boxed areas. **(F)** Representative IFA images of myosinX in DAOY cells on collagen-coated plates -/+ bFGF for 18 h. **(G)** Representative IFA images of myosinX in DAOY spheroids embedded in collagen -/+ bFGF for 18 h. Magnifications are 4x of boxed areas. Yellow arrowheads indicate myosinX-positive filopodia.

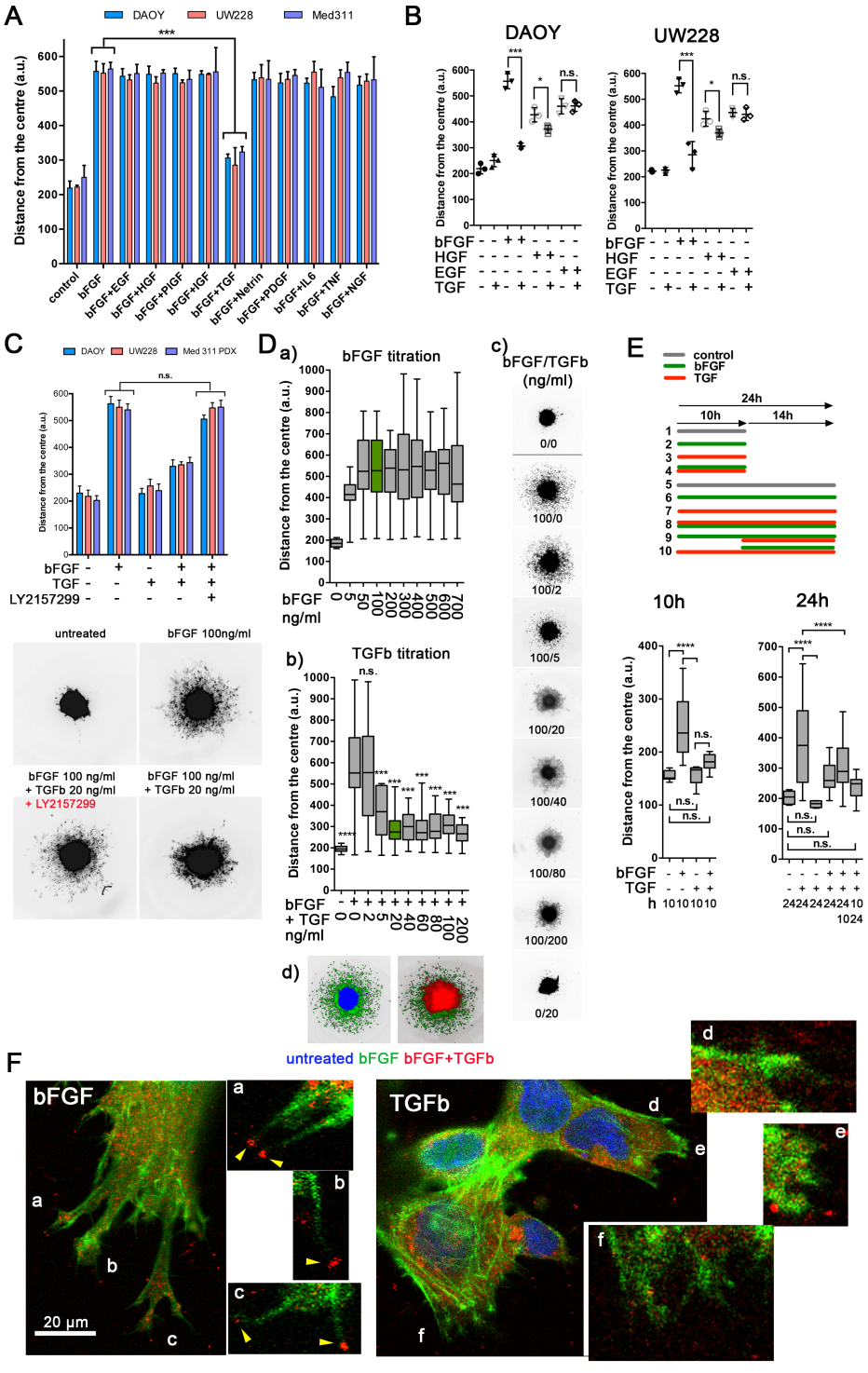


Figure 3: TGF-β antagonizes bFGF-induced cell motility and invasiveness. (A) Quantification of SIA after 24 h in DAOY, UW228 and Med311PDX cells (n=3, means ± SD). Individual growth factors (EGF (30 ng/ml), HGF (20 ng/ml), IGF (20 ng/m), Netrin (200 ng/ml), PDGF-B (20 ng/ml), TNF –α (25 ng/ml), IL-6 (20ng/mL), NGF (50 ng/mL), PlGF-1 (10 ng/mL) and TGF-β (20 ng/mL)) were combined with bFGF (100 ng/ml). **(B)** SIA using combinations of bFGF (100 ng/ml), EGF (30 ng/ml), HGF (20 ng/ml) with TGF-β (20 ng/ml) for 24h in DAOY and UW228 cells (n=3, means ± SD). **(C)** DAOY, UW228, Med311PDX cells treated with bFGF + TGF-β -/+ LY2157299 for 24h. Rep. images of spheroids after 24 h. **(D)** Quantification of SIA (pooled data of two independent experiments). a) bFGF titration in DAOY cells. b) TGF-β titration in bFGF-stimulated DAOY cells. c) Rep. images of b). d) Overlay of images of DAOY spheroids treated with -/+ bFGF (100 ng/ml) or bFGF (100 ng/ml) + TGF-β (20 ng/ml) for 24 h. **(E)** (Top) Diagram depicting the treatment schemes for SIA using DAOY cells with bFGF or TGF-β treatment for 10 h followed by co-stimulation with bFGF and TGF-β for 14 hours. (Bottom) Quantification of resulting SIA. Box plots with pooled data of two independent experiments are shown. **(F)** IFA as described for figure 2G except that the cells were also treated with TGF-β.

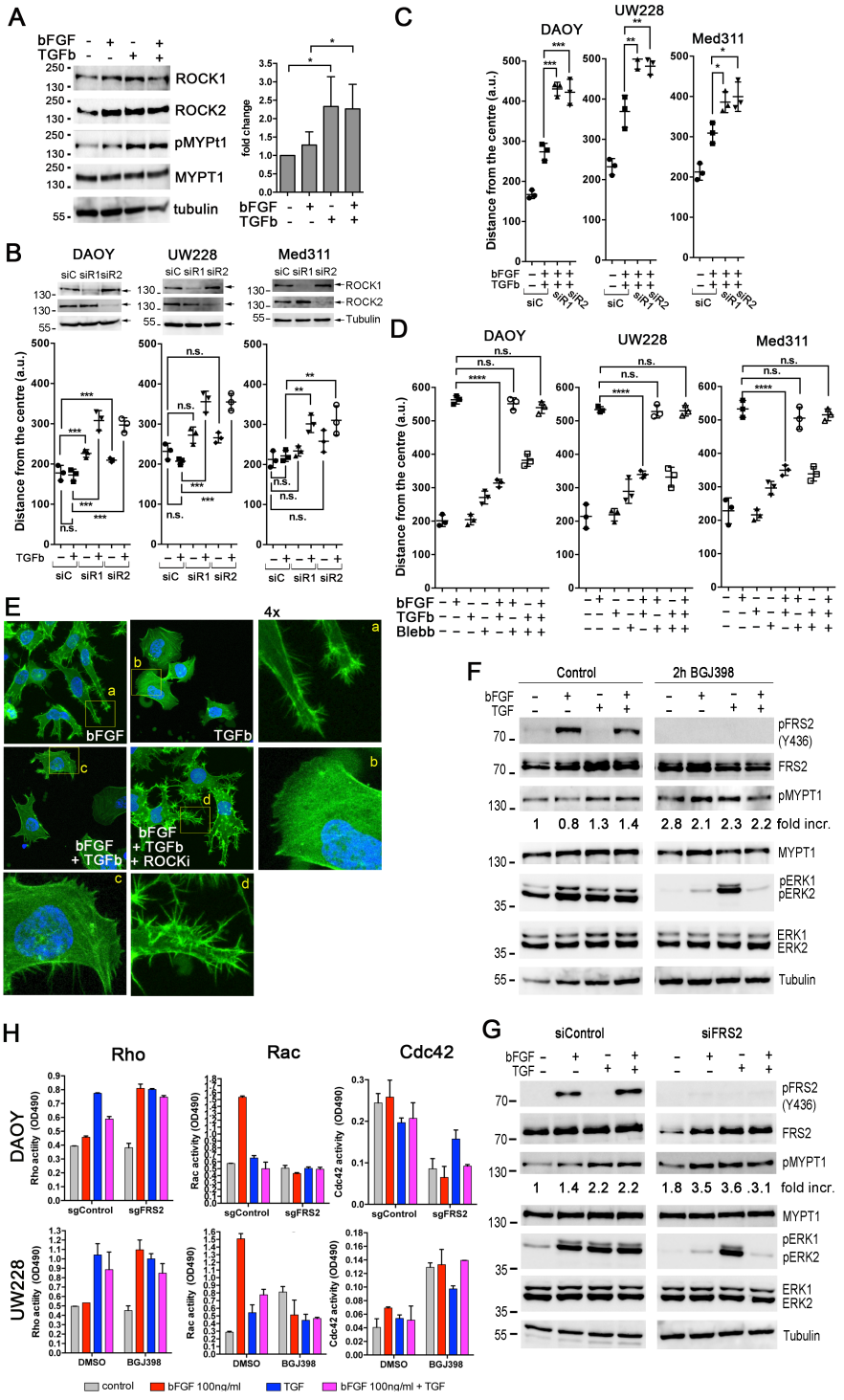


Figure 4: TGF-β causes ROCK activation and represses filopodia formation. (A) IB of DAOY cell lysates after stimulation with bFGF, TGF-β and bFGF + TGF-β for 10 minutes. Quantification of fold change of phospho MYPT1. **(B)** IB of DAOY, UW228 and Med311PDX cell lysates 72 h after siROCK1 and siROCK2 transfection. Quantification of SIA (n=3, means ± SD). Comparison of DAOY, UW228 and Med311PDX cells transfected with siROCK1 or siROCK2, +/- TGF-β stimulation for 24 h. **(C)** Same as (B) except that cells were also treated with bFGF. **(D)** Quantification of SIA of DAOY, UW228, Med 311 PDX cells treated with TGF-β +/- blebbistatin for 24h (means ± SD, n=3). **(E)** High resolution merged z-stacks of SIA of DAOY cells treated with GF and ROCKi Y27632 for 24 h. **(F)** IB of DAOY cell lysates after treatment with +/- BGJ398 and stimulation with GFs for 10 minutes. Fold change of phospho MYPT1 is indicated below the pMYPT1 panels. **(G)** IB of DAOY, UW228 and Med311PDX cell lysates after transfection with siFRS2 or non-targeted siRNA and after stimulation with GFs for 10 minutes. **(H)** Quantification of activity of Rho, Rac and Cdc42 using sgControl, sgFRS2 and UW228 cells treated with +/- BGJ398 after stimulation with GFs for 24 h.

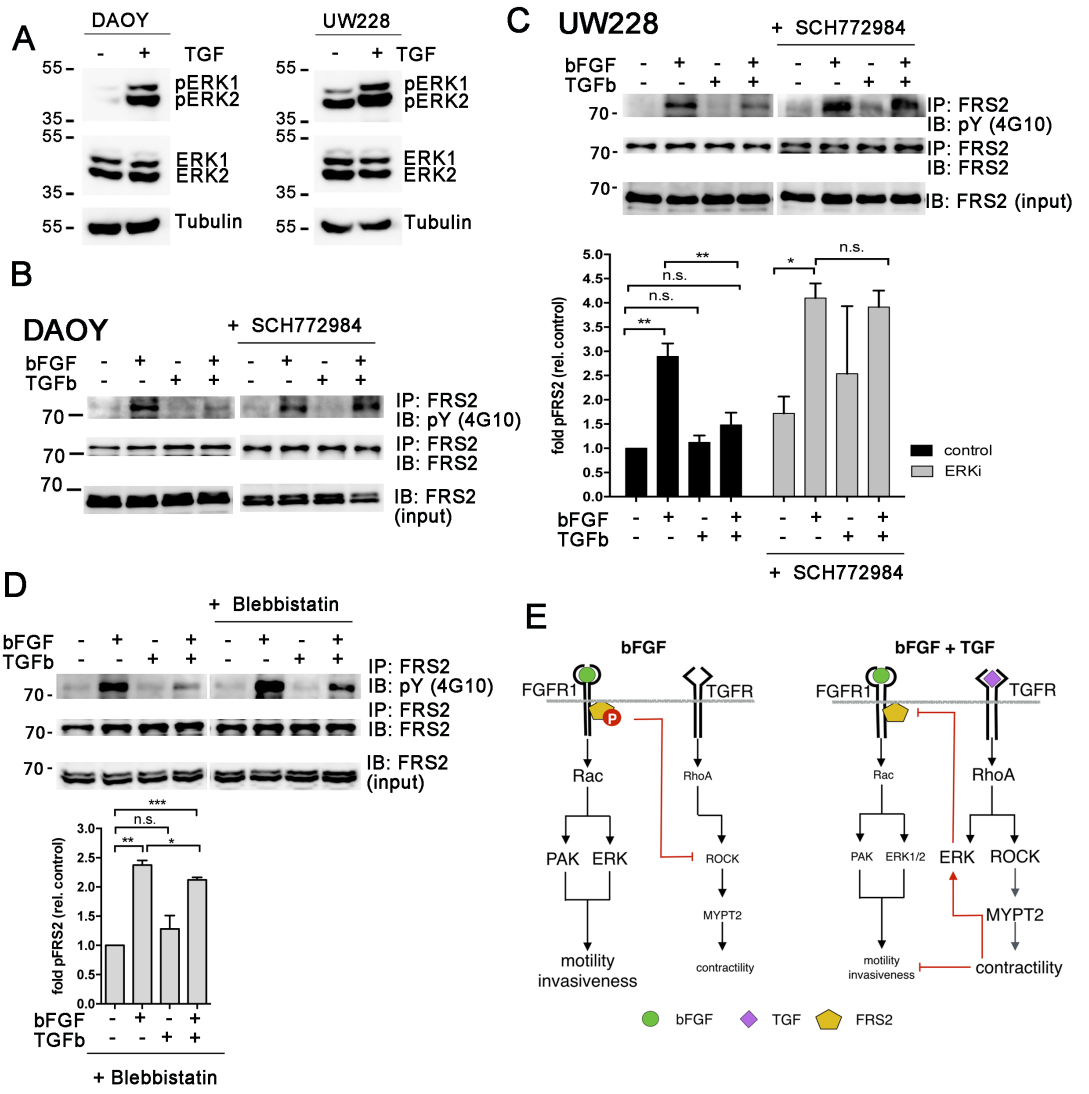


Figure 5: TGF-β-induced ERK represses FRS2 activation (A) IB of DAOY and UW228 cell lysates after stimulation with TGF-β for 10 minutes. **(B)** DAOY cells treated with -/+ SCH772984 for 2 h followed by stimulation with bFGF, TGF-β, or co-stimulation with bFGF and TGF-β for 10 min were subjected to anti-FRS2 IP followed by IB for total tyrosine phosphorylation (4G10). Quantification of fold change phospho FRS2 relative to control. **(C)** Same as (B) using UW228 cells. **(D)** Same as (B) except the cells were treated with -/+ blebbistatin. **(E)** Schematic representation of the inhibition of ROCK by FRS2 in the absence of TGF-β and inhibition of FRS2 by ERK-induced TGF-β.

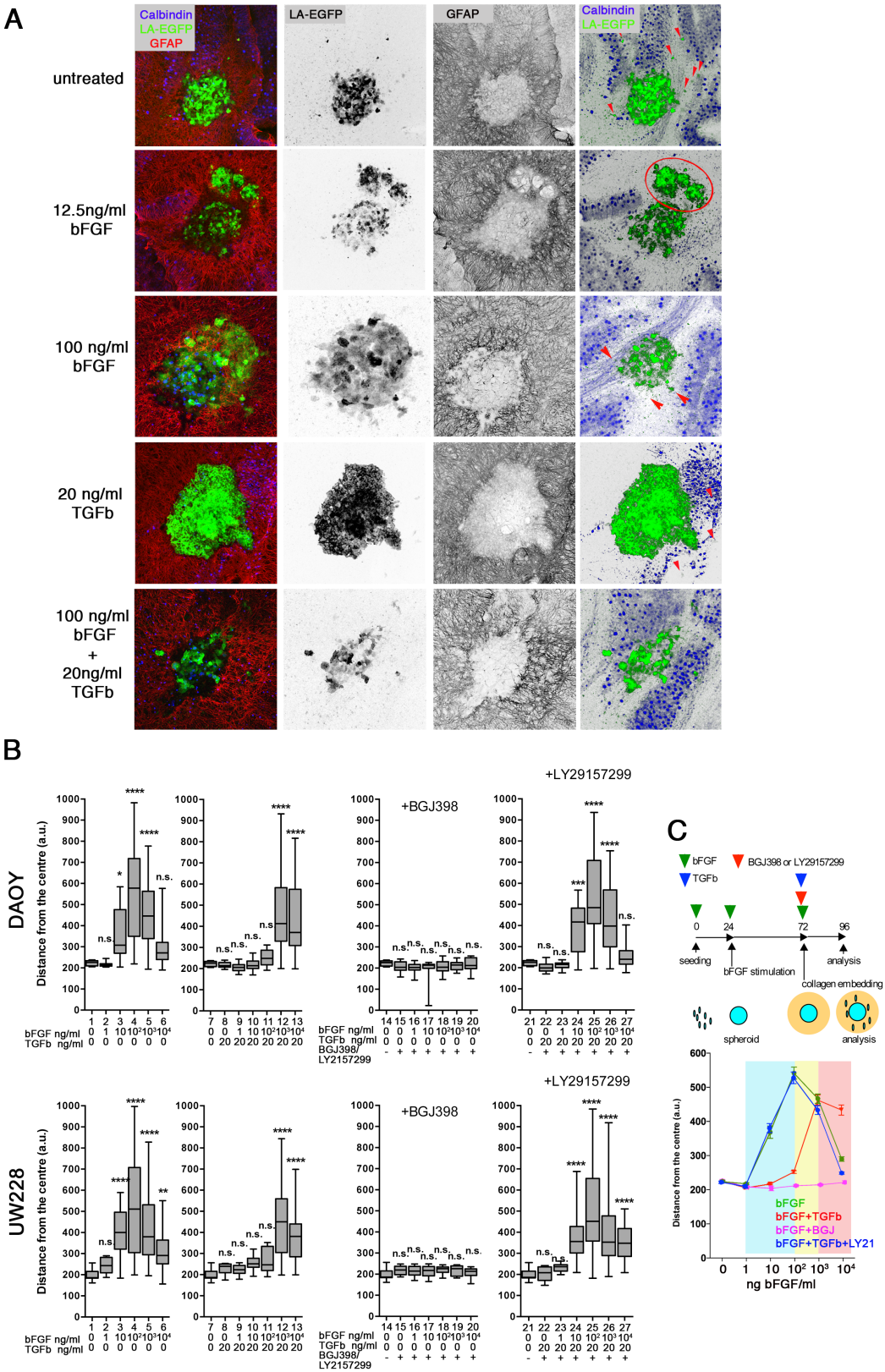


Figure 6: TGF-β rescues migration under high bFGF conditions (A) Confocal images of OCSC and DAOY spheroids co-culture for 5 days either untreated or with bFGF (*ex vivo* low), bFGF (*ex vivo* high), TGF-β or bFGF (*ex vivo* high) + TGF-β. **(B)** Quantification of SIA (pooled data of two independent experiments) using DAOY and UW228 cells after treatment with bFGF (1 ng/ml to 10 µg/ml) -/+ TGF-β in combination with BGJ398 or LY29157299. **(C)** (Top) Treatment schemes used in B. (Bottom) Graphical representation of outcome of (B).

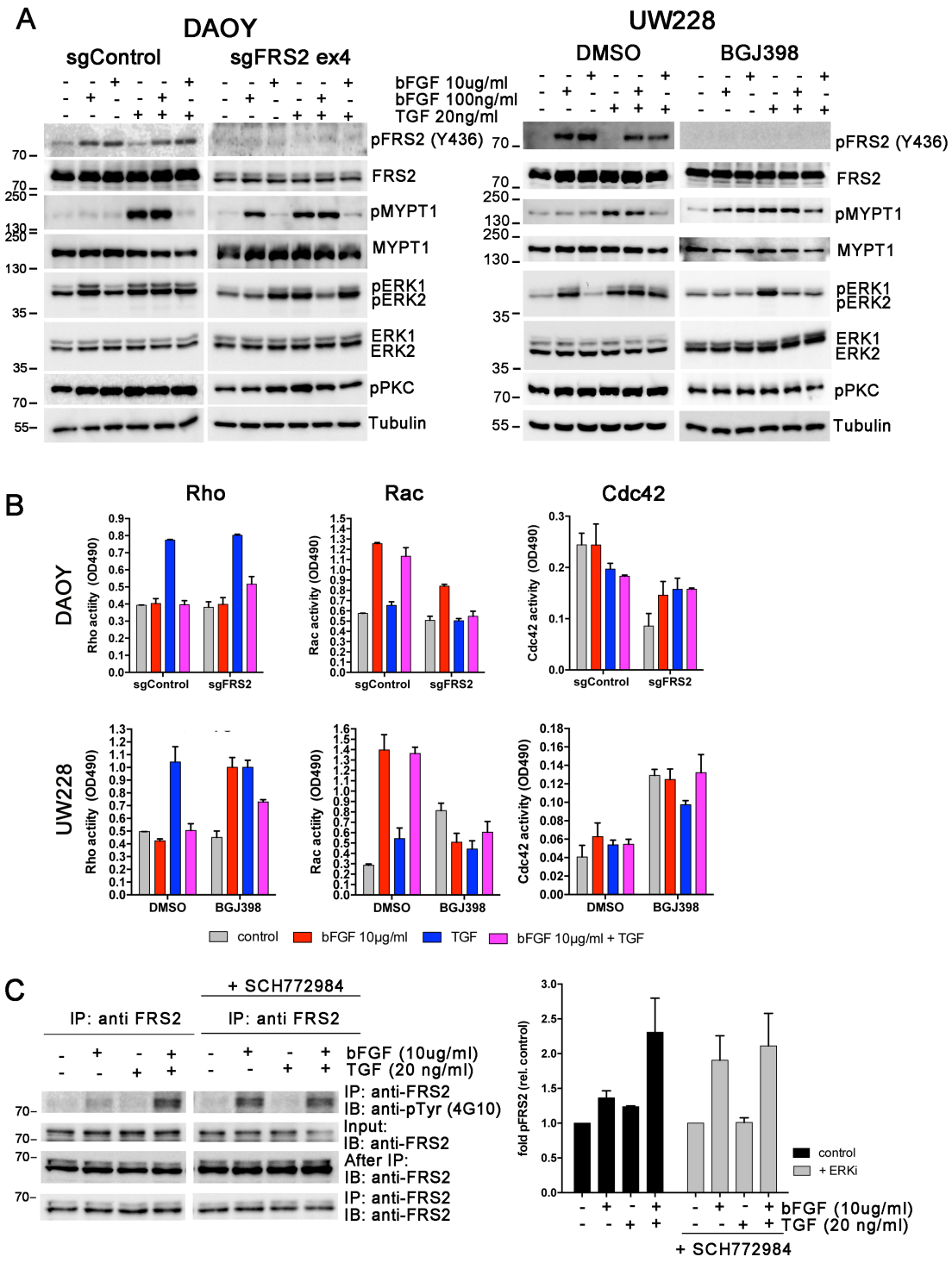


Figure 7: TGF-β reverts negative feed-back regulation under high bFGF conditions (A) IB of cell lysates of collagen-embedded DAOY sgControl, DAOY sgFRS2 ex4 and UW228 after stimulation with bFGF (*in vitro* low), bFGF (*in vitro* high), TGF-β, bFGF (*in vitro* low) + TGF-β or bFGF (*in vitro* high) + TGF-β -/+ BGJ398 for 24 h. **(B)** Quantification of the activity of Rho, Rac and cdc42 using the cell lysates described in A. **(C)** IP anti-FRS2 IP followed by IB for total tyrosine phosphorylation of DAOY cells treated with -/+ SCH772984 and stimulated with bFGF, TGF-β, or co-stimulation with bFGF + TGF-β for 24 h. Bar graph shows fold change of FRS2 phosphorylation relative to untreated control.

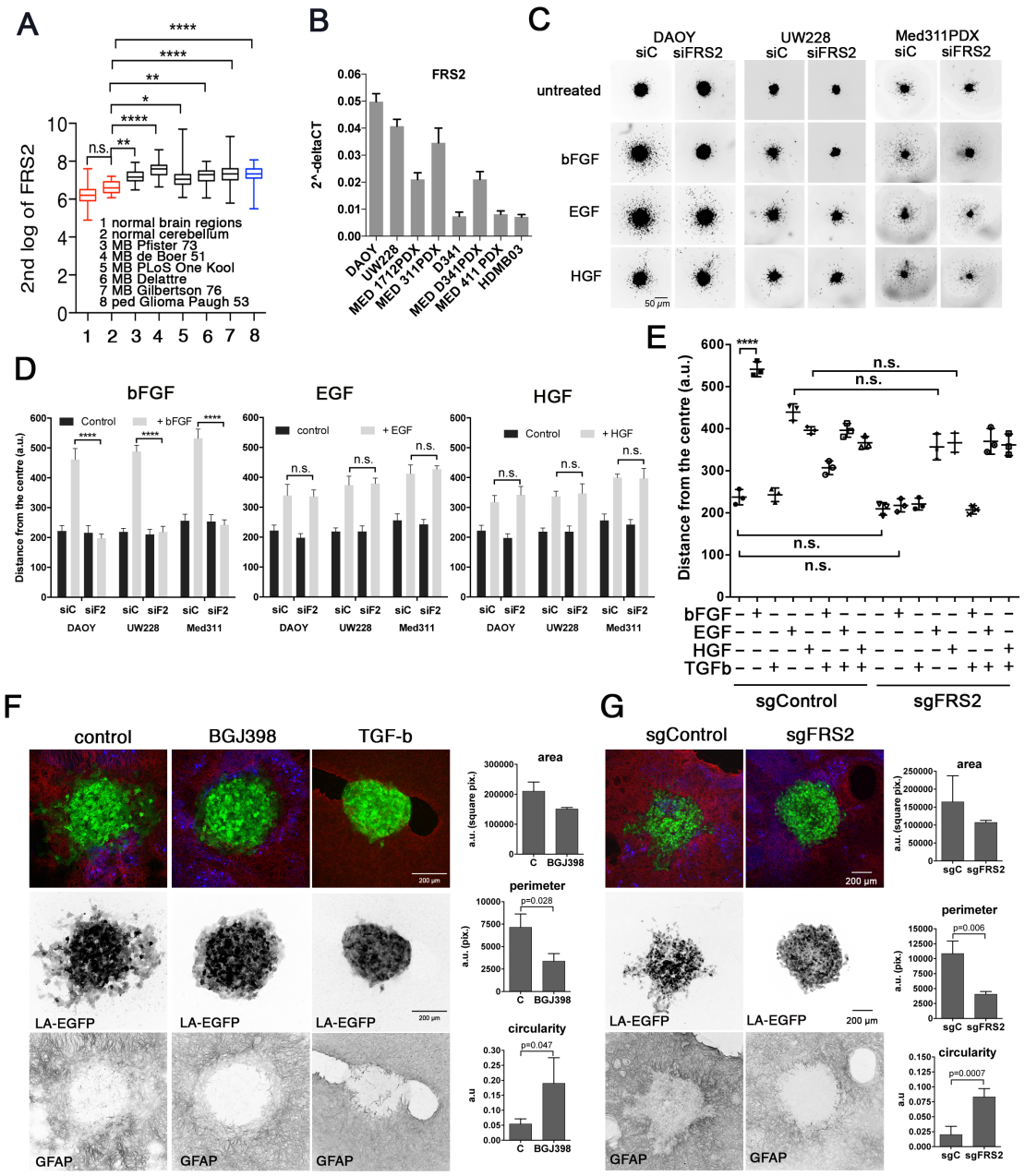


Figure 8: FGFR-FRS2 signaling promotes brain tissue infiltration (A) Comparative microarray data analysis for FRS2 mRNA expression. **(B)** FRS2 mRNA expression levels in various MB cell lines (n=3, means ± SD). **(C)** Representative images of DAOY, UW228 and Med311PDX spheroids transfected with siControl or siFRS2 and treated with bFGF, EGF (30 ng/ml) or HGF (20 ng/ml) for 24 h. **(D)** Quantification of SIA shown in (C) (n=3, means ± SD). **(E)** Quantification of SIA using DAOY sgControl and DAOY sgFRS2 cells after stimulation with bFGF, TGF-β, EGF (30 ng/ml) or HGF combinations for 24 h (n=3, means ± SD). **(F)** OCSCs co-cultured with DAOY LA-EGFP and either left untreated or after treatment with BGJ298 or TGF-β for 5 days. IFA anti GFAP (red) and Calbindin (blue). Quantification of the area, perimeter and the circularity of co-cultured DAOY spheroids (n=3, means ± SD). **(G)** OCSCs with DAOY sgControl and DAOY sgFRS2 spheroids after 5 days of co-culture. Quantifications as in (F).

Manuscript 3: Supplementary information

Experimental Procedures:

Reagents:

Growth factors were used throughout the study in the concentrations indicated. basic Fibroblast Growth Factor (bFGF, 100-18B): 100 ng/ml (low, *in vitro*), 10 µg/ml (high, *in-vitro*), 12.5 ng/ml (low, OCSC), 100 ng/ml (high, OCSC) , Transforming Growth Factor-β (TGF-β, 100-21): 20 ng/ml, Hepatocyte growth factor (HGF, 100-39): 20 ng/ml, epidermal growth factor (EGF, 100-47): 30 ng/ml from PeproTech (London, UK). The following growth factors/cytokines were used for PB design: Netrin (R&D Systems, 6419-N1-025), HGF, EGF, Insulin like Growth Factor 1 (IGF, 100-11), Platelet Derived Growth Factor-B (PDGF-B, P100-14B), Tumor Necrosis Factor-α (TNF- α, 300-01A) bFGF, Interleukin-6 (IL-6, 200-06), Nerve Growth Factor-β (NGF, 450-01), TGF-β and Placental Growth Factor-1 (PlGF-1, 100-06) from PeproTech (London, UK). For 'high' and 'low' levels of the respective growth factors refer to Table 2. Inhibitors were used throughout the study in the concentrations as indicated in Suppl. Figure 2A, unless specified otherwise. ML141 (S7686), SCH772984 (S7101), BGJ398 (S2183), SP600125 (S1460), U0126 (S1102), LY294002 (S1105), IPA-3 (S7093), Go6983 (S2911), NSC23766 (S8031), CCG-1423 (S7719), Y-27632 (S1049), LY2157299 (S2230) were purchased from Selleckchem, Houston, TX, USA. H-1152 (ALX-270-423, Alexis Biochemicals), CK666 (SML0006, Sigma Aldrich), blebbistatin (B0560, Sigma Aldrich).

Antibodies:

Primary Antibodies were used in the dilutions as mentioned in Table 1. Anti-bFGF (sc-79) from Santa Cruz Biotechnology, anti-FGFR1 (ab10646), anti-GFAP (ab53554) and anti-calbindin (ab11426) from Abcam, anti-phospho-ERK1/2 (9101), anti-ERK1/2 (9102), anti-phospho-FRS2 (Y436) (3861S), anti-ROCK1 (4035), anti-phospho-PKC (pan βII Ser660) (9371), anti-MYPT1 (2634), anti-mouse horseradish peroxidase (HRP)-linked (7076) and anti-rabbit HRP linked (7074) from Cell signaling Technology, anti-FRS2/SNT-1 (05-502), anti-ROCK2 (ABS436), anti-phospho-tyrosine (4G10® platinum) (05-1050) and anti-phospho MYPT1 (Thr696) (ABS45) from Merck Millipore, anti-MyosinX (HPA024223) and anti-tubulin (T9026) from Sigma-Alrich and anti-rabbit-Cy3–coupled (711-165-152) anti-mouse-Cy5–coupled (415-175-166) from Jackson Immuno Research.

Cultivation of human MB cell lines and patient-derived xenograft (PDX) cells culture:

Med-1712FH and Med-411FH cells were cultured in neurobasal medium (Invitrogen/Life Technologies, Paisley, UK, 12349-015) supplemented with 2% B-27® (Gibco/Life Technologies, 10889-038), 1% L-Glutamine (Invitrogen/Life Technologies, 25030024), 10 µg/ml bFGF (100-18B, PeproTech, London, UK) and 10 µg/ml EGF (100-47, PeproTech, London, UK). Med311PDX cells were cultured in NeuroCult NS-A Basal Medium (Human) with NeuroCult NS-A proliferation supplements (Human) (05751, Stem Cell Technologies), 10µg/ml bFGF (100-18B, PeproTech, London, UK), 10µg/ml EGF (100-47, PeproTech, London, UK) and 1% Penicillin/Streptomycin (15140122, Gibco by Life Technologies) on laminin (L2020-1MG, Sigma-Aldrich, dilute 1mg/ml laminin (1:100) in PBS) coated tissue culture

treated dishes.

Immunohistochemistry (IHC):
IHC of MB Tissue Microarray (TMA) and normal brain sections was performed by Sophistolab (Muttenz, Switzerland) on a Lecia BondMax instruments using Refine HRP-Kits (Leica DS9800).All buffer-solutions were purchased from Lecia Microsystems Newcastle, Ltd and used according to the manufacturer's guidelines. Paraffin-slides were de-waxed, pre-treated and incubated as follows: ER-solution 2 for 10 minutes at 95°C, ER-solution 2 for 20 minutes at 100°C and ER-solution 2 for 30 minutes at 100°C. The TMA slides were captured digitally using Axio Observer 2 mot plus fluorescence microscope (Zeiss, Munich, Germany). The expression of bFGF and FGFR1 was assessed independently at 5x to 20x magnifications. The samples in the TMA slides were classified by H scores by the assessor who was blind to the clinicopathological data of the patients as high, moderate, low and negative expression of bFGF and FGFR1.

Expression analysis using R2 database:
Expression of FRS2 in normal brain, normal cerebellum and MB samples were analyzed using the open access platform R2 for visualization and analysis of the microarray data (http://r2.amc.nl) as described in (Kumar et al., 2015b). The following datasets were used: Normal brain regions – 172 – MAS5.0 – u133p2 (172 samples, postmortem brain tissue collected from ADRC brain banks), Normal cerebellum – Roth – 9 – MAS5.0 – u133p2 (9 samples), MB (SHH) Pfister – 76 – u133p2 (73 pediatric MB samples), MB ependymoma – denBoer – 51 – u133p2 (51 samples), MB PLoS One – Kool – 62 – MAS5.0 – u133p2 (62 human MB tumor samples), MB public – Delattre – u133p2 (57 samples), Tumor MB – Gilbertson – 76 – u133p2 (76 samples) and Tumor Glioma pediatric – Paugh – 53 – u133p2 (53 samples).

RNA expression analysis by qRT-PCR:
Total RNA was isolated using RNeasy Mini Kit (Qiagen, Basel, Switzerland) following the manufacturer's instructions. 100 ng of total RNA was used as a template for reverse transcription, which was initiated by random hexamer primers. The cDNA synthesis was carried out using High capacity cDNA Reverse Transcription Kit (Applied Biosystems). qRT-PCR was performed under conditions optimized for the ABI7900HT instrument, using TaqMan® Gene Expression Master Mix (4369016, Applied Biosystems). Primer probes specific for the following genes (4331182, Applied Biosystems) were used: FGFR1 (Hs00915142_m1), FGFR2 (Hs01552926_m1), FGFR3 (Hs00179829_m1), FGFR4 (Hs01106908_m1), FGF2 (Hs00266645_m1), TGF-β1 (Hs00998133_m1), TGFBR1 (Hs00610320_m1), TGFBR2 (Hs00234253_m1) and TGFBR3L (Hs00418521_m1). Cycle threshold (CT) values were normalized to housekeeping gene GAPDH (Hs02758991_g1, Applied Biosystems). ΔΔCT method was used to calculate the relative gene expression of each gene of interest.

Immunoblotting (IB):
RIPA buffer lysates were resolved by SDS-PAGE and transferred to a nitrocellulose membrane using a transfer apparatus according to the manufacturer's instructions (Bio-

Rad). Membranes were probed with primary antibodies against phospho-FRS2, FRS2, ERK1/2, phospho-ERK1/2, MYPT1, phosphor-MYPT1, phospho-PKC and tubulin. HRP-linked secondary antibodies (1:5000) were used to detect the primary antibodies. Chemiluminescence detection was performed using ChemiDoc Touch Gel and Western Blot imaging system (BioRad). Integrated density of Immuno-reactive bands was quantified using Adobe Photoshop CS3.

RNA interference:

Approximately 75% confluent cells were transfected with siRNA specific for ERK1 (MAPK3 – ID: s11140), ERK2 (MAPK1 – ID: s11137), ROCK1 (ID: s12097), ROCK2 (ID: s18163), FRS2 (ID: s21261) or Silencer select negative control (ID: 4390843, Ambion). Each siRNA was used at a final concentration of 5 nmol along with DharmaFECT 4 transfection regent (T-2004-03, Dharmacon). After 48 hours, RNA and proteins were isolated from the cells to determine gene expression by qRT-PCR and protein expression by IB. On successful down-regulation of the protein of interest, the transfected cells were used for SIA.

Immunofluorescence:

Single cells (2D): 8 well ibidi plates were coated with collagen (1:10 in 60% EtOH) and left in the incubator at 37°C overnight. The following day, approximately 500 cells / 200 µl per well is seeded on the collagen coated ibidi plates. 24 hours after seeding, the medium was removed and the cells were treated with bFGF for 10 h. The cells were fixed and treated as described in (Ma and Baumgartner, 2014). The fixed cells were incubated with diluted primary antibodies (refer table 1 for dilutions) overnight at 4°C. Anti-rabbit-Cy3 – (711-165-152) coupled secondary antibody was diluted 1:300 and incubated for 2 hours at RT. Z-stacks of the cells were acquired using a 63X immersion objective in SP8 Leica confocal microscope.
Spheroids (3D): DAOY LA_GFP cells were used to perform SIA in 8 well ibidi plate as described in (Kumar et al., 2015a). The embedded spheroids are treated with bFGF, TGF-β, bFGF+TGF and / or Y27632 for 24 hours at 37°C. After appropriate incubation, the medium is removed from the wells without disturbing the collage layer. The spheroids embedded in collagen is fixed with 4% ice cold PFA and permeabilized with 0.5% Triton-X-100 for 5 minutes. The spheroids were subsequently stained with Hoechst 1:5000 (B2883, Sigma-Alrich) and mounted with glycergel (Dako, C0563). Z-stacks of the invaded cells were acquired as described above (Confocal imaging – SIA).
Organotypic cerebellar slice culture (OCSC): The slice-spheroid co-cultures were stained for GFAP and calbindin as described in (Neve et al. 2017, submitted) and four-color image acquisition was performed on a SP8 confocal microscope.

G-LISA:

The activity of Rac1, Cdc42 and RhoA in collagen-embedded cells were determined using the G-LISA Rac1 (BK128), Cdc42 (BK127) and RhoA (BK124) G-LISA activation kits (Cytoskeleton, Inc.), respectively. 2 million cells/ml per well were seeded in 6 well cell repellent plates (657970, Greiner Bio-one®) and incubated overnight at 37°C. Cell clusters were embedded in collagen I (final concentration 3 mg/ml) (5005-B, Cellsystems). Fresh medium was added to the cells after polymerization of collagen I. DAOY sgControl and DAOY sgFRS2 collagen-embedded cell clusters were treated with bFGF (*in vitro low: 100 ng/ml, in vitro high 10*

µg/ml), TGF-β (20 ng/ml), and various combinations thereof for 24 h. UW228 cell clusters were treated as DAOY cells described above without or with BGJ398 for 24 h. Clusters were lysed and the total protein was isolated as described in (Keely et al., 2007). The levels of GTP-loaded Rac1, Cdc42 and RhoA in the lysates were determined by G-LISA activation kit.

G-LISA – 3D, IB:
Total protein was isolated from the collagen embedded cell clusters as explained above (G-LISA). The lysates were boiled with the loading buffer (Roti® - Load1, K929.1, Carl Roth, Germany) and analyzed by IB as described above.

Immunoprecipitation:
Serum starved cells were incubated with bFGF (*in vitro* low), TGF-β, bFGF (*in vitro* low) + TGF-β and / or SCH772894 for 10 min or bFGF (*in vitro* high), TGF-β, bFGF (*in vitro* high) + TGF-β and / or SCH772894 for 24 h. The cells were lysed with 1% NP40 lysis buffer without SDS and the protein concentrations were normalized among the samples. Anti-FRS2 antibody is incubated with 100 µl of Dynabeads® Protein G beads (10003D, Thermofischer Scientific) and 200 µl of PBST for 30 min on a eppendorf rotator at RT. The antibody-bead complex is isolated using the immunoprecipitation magnet (Merck Millipore). The equalized lysates were incubated with anti-FRS2-bead complex for 1 to 2 h at 4°C. The immune complexes were eluted from the beads by boiling the samples with the loading buffer (Roti® - Load1, K929.1, Carl Roth, Germany) and were analyzed by IB as described above.

PB design:
Plackett Burman (PB) design, an orthogonal screening matrix that yields unbiased estimates of all independent variables in the smallest design possible, will be adapted to perform the combinatorial growth factor screen (Plackett and Burman, 1946). Multiple growth factors affect MB cell dissemination simultaneously. PB is based on Two-level (High and Low) Hadamard screening matrices where 'n' variables (number of growth factors to be tested = 11) can be tested in 'n+1' runs. The 11 growth factors that were tested in combinations of high and low levels were as follows: HGF, EGF, Netrin, TNF-α, IGF, PDGF-B, TGF-β, IL-6, bFGF, NFG and PlGF-1. The PB screening matrix for 12 runs is shown in Fig. S6A. The high levels of the growth factors / cytokines are represented as "+" and those in low levels are represented as "-". The high and low levels of the 11 growth factors / cytokines screened were determined by 1:2 serial dilution. The minimum concentration of the growth factor required to induce a measureable level (statistically significant) of cell dissemination as compared to the control was set as the low level of the growth factor / cytokine.

Organotypic cerebellar slice culture:
Organotypic cerebellar slice culture was set-up and the tumor spheroid formation and initiation of co-culture was performed as described in (Neve et al. 2017, submitted). Tumor spheroids were formed with DAOY LA-GFP cells, DAOY sgControl cells and DAOY sgFRS2 cells. The co-culture was treated with bFGF (OCSC low and OCSC high), TGF-β, bFGF (OSCS high) + TGF-β and / or BGJ398. Spheroids were incubated for 7 or 5 days for DAOY sgControl and DAOY sgFRS2 or control and BGJ398-treated, respectively. Following the treatment, the co-cultures were fixed as described in (Neve et al. 2017, submitted). The fixed co-cultures

were stained and analyzed using immunofluorescence techniques as explained above (Immunofluorescence OCSC).

LentiCRISPR:

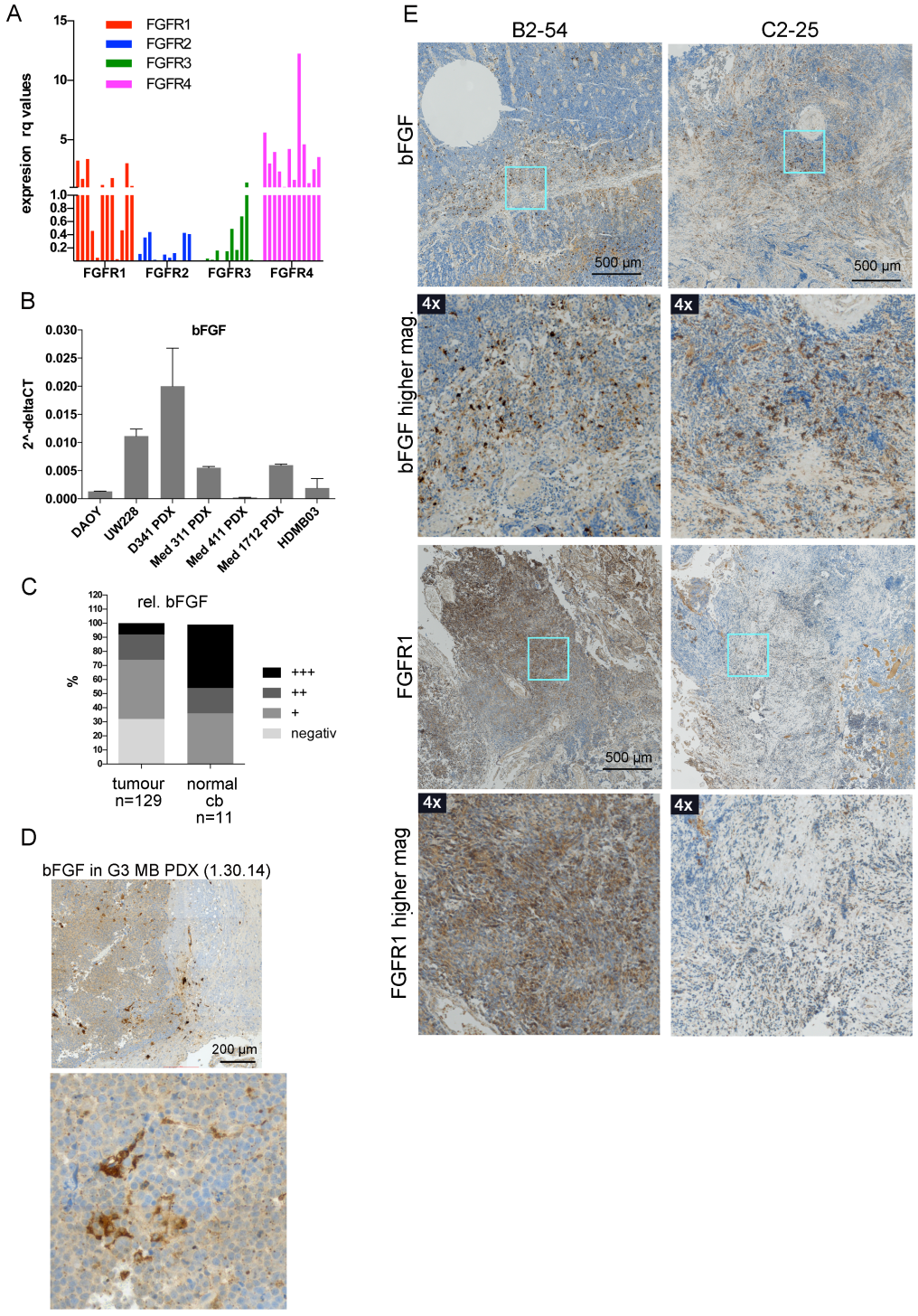
BFP tagged LentiCRISPR plasmids were generously provided by Dr. Scott McComb (Ottawa, Canada). Cloning of sgRNA into the LentiCRISPR plasmids were performed with a single-tube restriction and ligation method as described in (McComb et al., 2016). Production of lentiviral vectors was performed according to the standard protocol. In brief, 293T cells were transfected with using HEPES-buffered saline solution (HeBS) and 0.5 M calcium phosphate with LentiCRISPR, pVSV, pMDL, and pRev (Kindly provided by Dr. Oliver Pertz, Bern, Switzerland) in a ratio of 4.5:1.5:3:1. The media was changed after 12 h, and the virus was collected at 72 h after transfection of plasmids. Viral transductions were performed using hexadimethrine bromide (H9268, Sigma-Aldrich). sgRNA sequences were screened for FRS2 activity in DAOY cells by IB. The most effective sequence was chosen for further experiments. The specific sg target sequences used are listed below:

Gene	**Exon**	**Sg target sequence**
FRS2	Exon1	AACTTGTTCCGATGGTTATCTGG
FRS2	Exon2	TACCTCTGCCTGCGACGCTATGG
FRS2	Exon3	TAGGTGTTCGAGGTGTTCTAGGG
FRS2	Exon4	AGGATGTCTGCTTGACGGATGGG

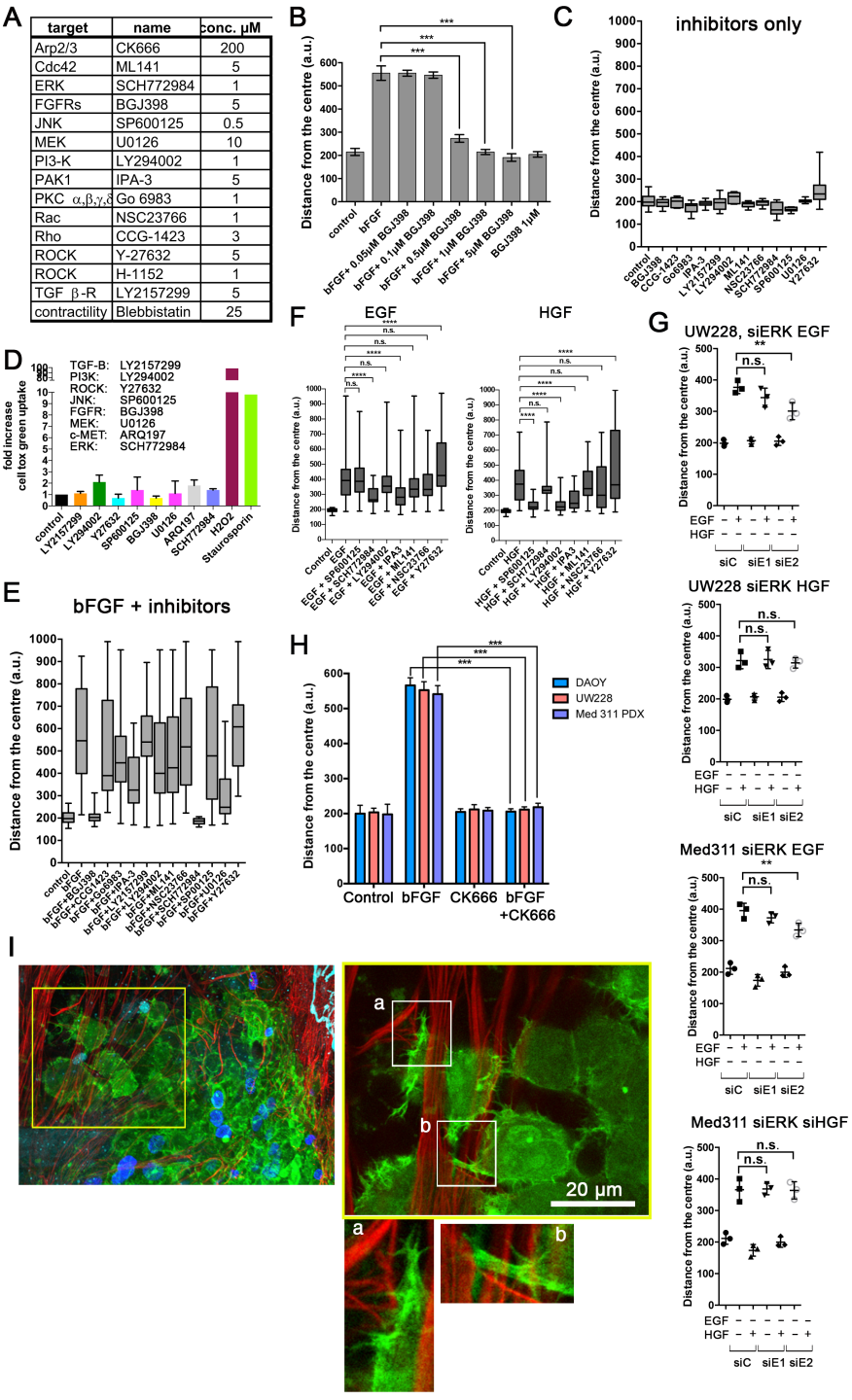
Statistical Analysis:

Mean ± SD are shown when means of three independent experiments are compared, and box plots with whiskers to min and max are shown when multiple individual measurements from two to three independent experiments are compared. Unpaired student's t-test was used to test significance of differences between two samples acquired in three independent experiments. For all other analyses one-way ANOVA repeated measures test using Bonferroni's Multiple Comparison was performed. *P*-Values < 0.05 were considered significant (* $p \leq 0.05$, ** $p \leq 0.01$, *** $p \leq 0.001$), **** $p \leq 0.0001$). Where indicated, asterisks show statistical significances between control and test sample.

Supplementary Figures:



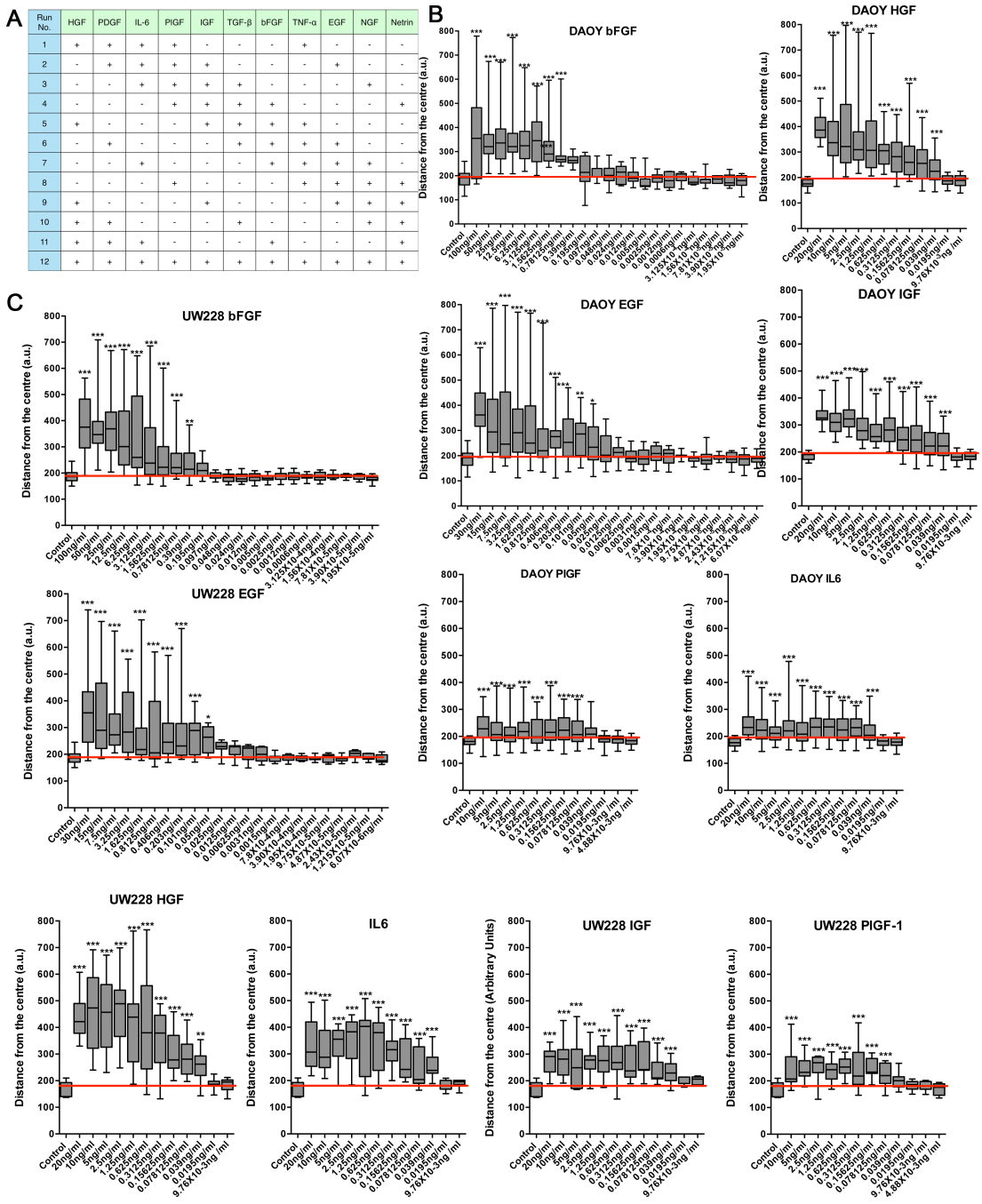
Suppl. Figure 1: MB tumor tissue is infiltrated by bFGF-producing cells. (A) qRT-PCR analysis of FGFR1-4 expression in a small cohort of MB tumors. **(B)** Comparative qRT-PCR expression analysis of bFGF in established MB cell lines. **(C)** Quantification of bFGF expression in MB TMA. Comparison of tumor and nearby normal cerebellar tissue. **(D)** Anti-bFGF IHC analysis of group 3 PDX. **(E)** Anti-bFGF (upper) and anti FGFR1 (lower) IHC analysis of primary MB validated clinical isolates. Magnifications are 4x of boxed areas.



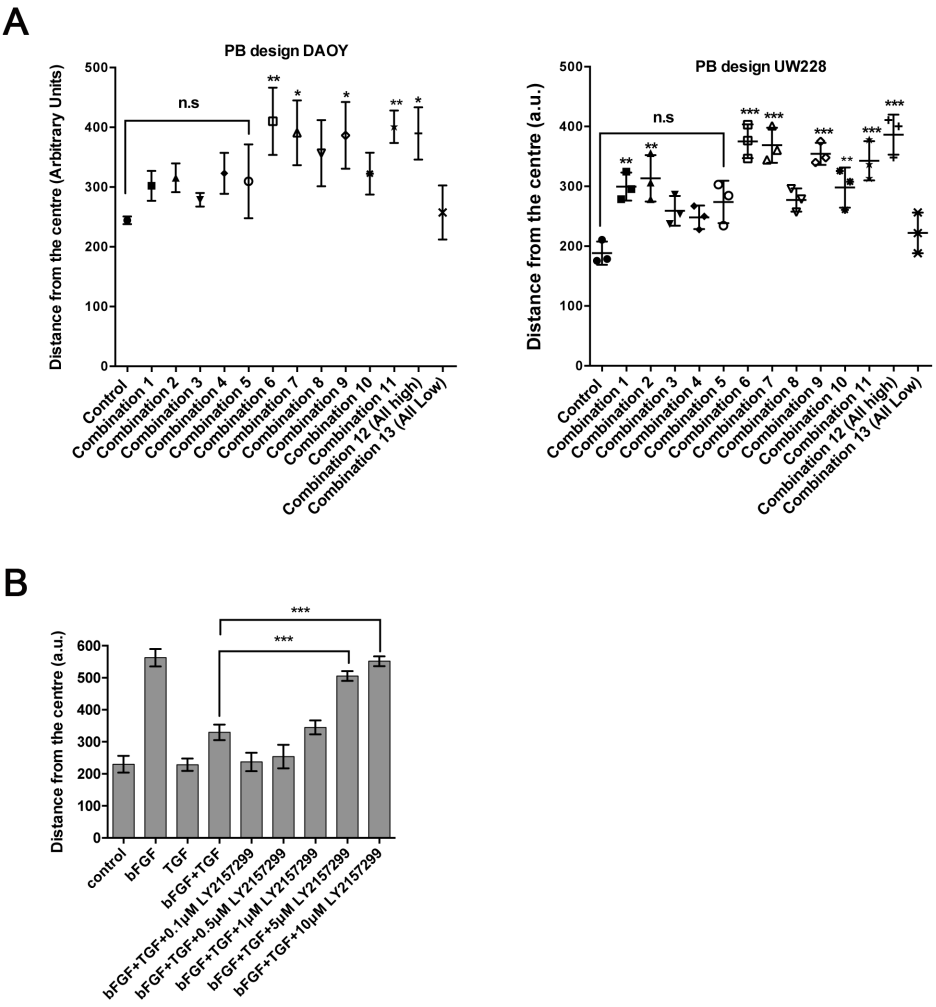
Suppl. Figure 2: bFGF promotes migration and tissue infiltration of MB cells (A) Concentrations of the various inhibitors used in this study. **(B)** Quantification of SIA using DAOY-LA-EGFP after treatment with BGJ398 + bFGF for 24 h (n = 3, means ± SD). **(C)** Quantification of SIA (pooled data of two independent experiments) using DAOY-LA-EGFP spheroids after 24 h treatment with inhibitors (BGJ398, CCG-1423, Go6983, IPA3, LY2157299, LY294002, ML141, NSC23766, SCH772984, SP600125, U0126 and Y27632) in concentrations as indicated in (A). **(D)** CellTox Green viability analysis of DAOY spheroids treated for 24 h with inhibitor concentrations indicated in (A) (n=3, means ± SD). **(E)** Quantification of SIA using DAOY spheroids after 24 h treatment with inhibitors at concentrations as indicated in (A) (pooled data of two independent experiments). **(F)** Quantification SIA using DAOY spheroids after 24 h treatment with inhibitors at concentrations as indicated in (A) in combination with HGF (20ng/ml) or EGF (30ng/ml). **(G)** Same as Figure 2C expect UW228 and Med311PDX cells were used. **(H)** Quantification of SIA using DAOY, UW228 or Med311PDX spheroids stimulated with bFGF -/+ CK666 for 24 hours (n = 3, means ± SD). **(I)** OCSC co-cultured for 15 d in the presence of 100 ng/ml bFGF. b and b are 4x magnifications of regions boxed.

A

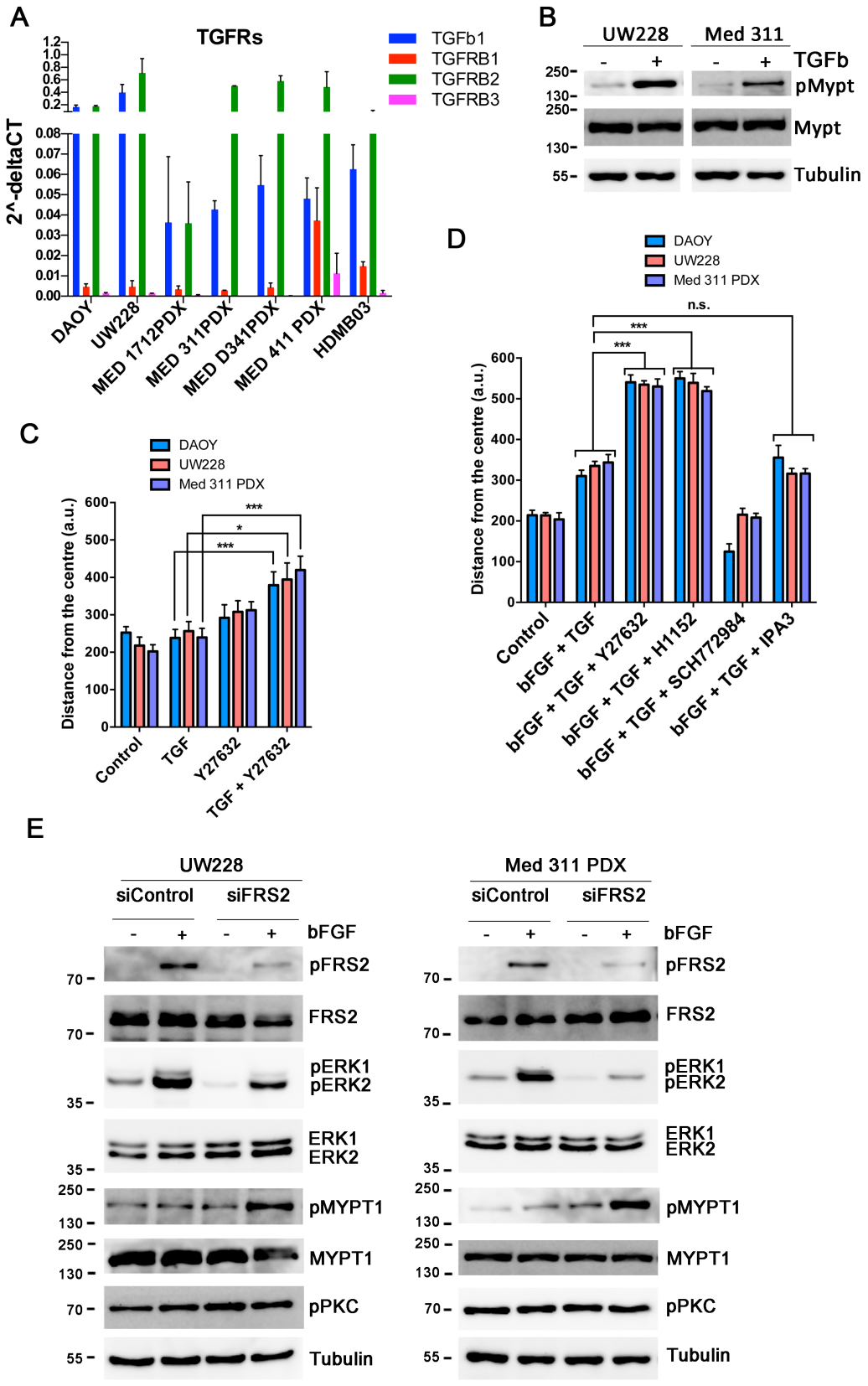
Run No.	HGF	PDGF	IL-6	PlGF	IGF	TGF-β	bFGF	TNF-α	EGF	NGF	Netrin
1	+	+	+	+	-	-	-	+	-	-	-
2	-	+	+	+	+	-	-	-	+	-	-
3	-	-	+	+	+	+	-	-	-	+	-
4	-	-	-	+	+	+	+	-	-	-	+
5	+	-	-	-	+	+	+	+	-	-	-
6	-	+	-	-	-	+	+	+	+	-	-
7	-	-	+	-	-	-	+	+	+	+	-
8	-	-	-	+	-	-	-	+	+	+	+
9	+	-	-	-	+	-	-	-	+	+	+
10	+	+	-	-	-	+	-	-	-	+	+
11	+	+	+	-	-	-	+	-	-	-	+
12	+	+	+	+	+	+	+	+	+	+	+



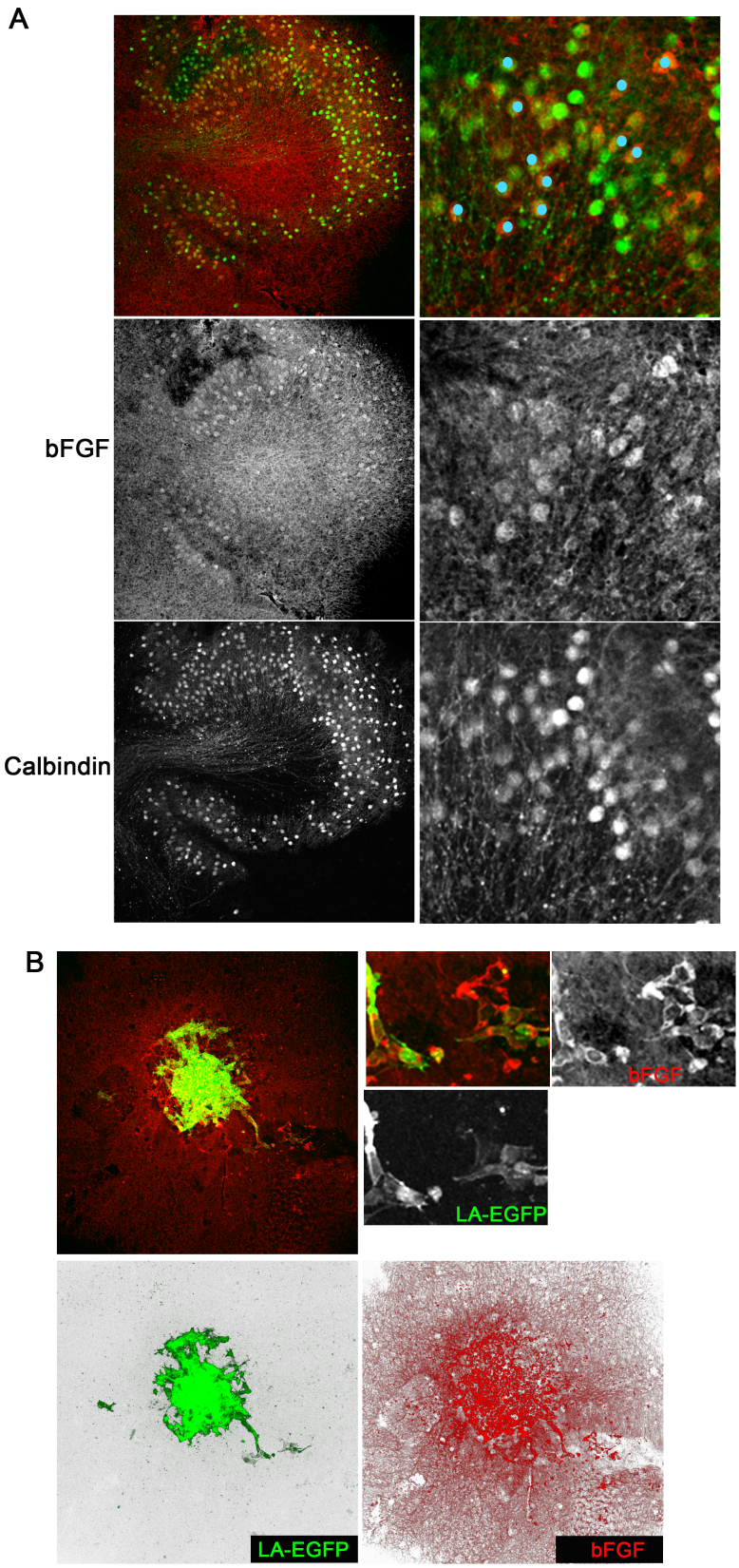
Suppl.Figure 3: (A) Plackett Burman screening (PB) matrix for 11 variables adapted for combinatorial GF screen. (+) = means high (causing maximal change), (-) = low (causing minimal significant change) concentration of GF. **(B)** Quantification of SIA of titrations of bFGF, HGF, EGF, IGF, PLGF-1 and IL-6 concentrations using DAOY cells (pooled data of two independent experiments). **(C)** Same as B in UW228 cells.



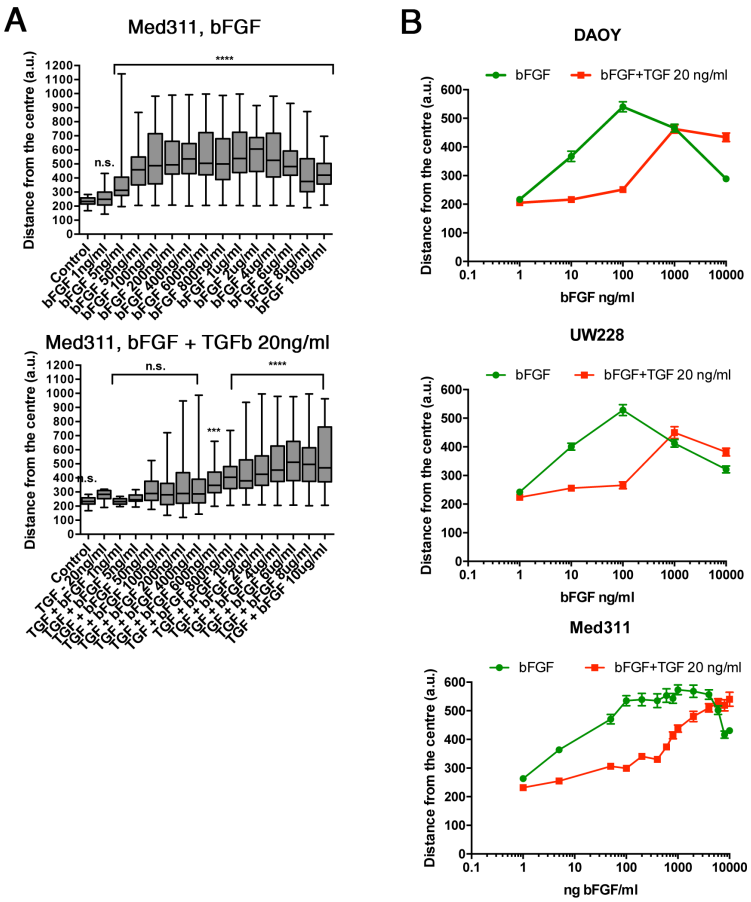
Suppl. Figure 4: TGFRβ inhibitor rescues migration and invasion in the presence of TGF-β (A) Quantification of SIA with PB growth factor screen using DAOY and UW288 cells (n=3, means ± SD). **(B)** Quantification of SIA using DAOY cells after stimulation with bFGF, TGF-β, bFGF + TGF-β or bFGF + TGF-β + increasing concentrations of TGFRβ inhibitor LY2157299 (n=3, means ± SD).



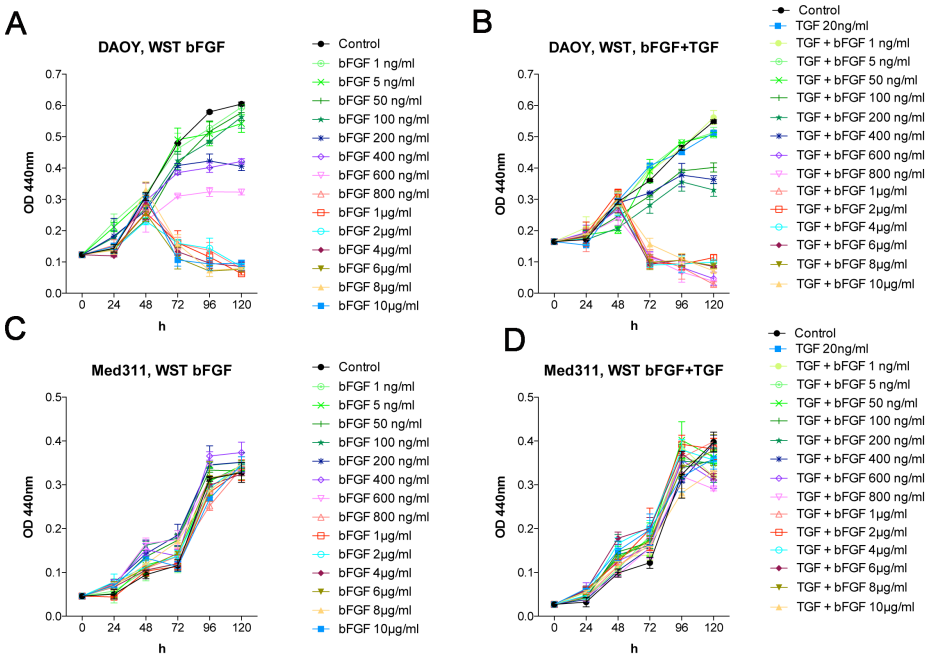
Suppl. Figure 5: (A) Comparative qRT-PCR expression analysis of TGF-β1, TGFRβ1, TGFRβ2 and TGFLRβ3 in established MB cell lines. **(B)** IB of UW228 and Med311PDX cell lysates after stimulation with TGF-β for 10 minutes. **(C)** Quantification of SIA using DAOY, UW228, Med311PDX cells stimulated with TGF-β -/+ CK666 for 24 hours (n=3, means ± SD). **(D)** Quantification of SIA using DAOY, UW228, Med311PDX cells stimulated with bFGF + TGF-β +/- Y27632, H1152, SCH772984 or IPA3 for 24 hours (n=3, means ± SD). **(E)** Same as Figure 4 (G) in UW228 and Med311PDX cells.



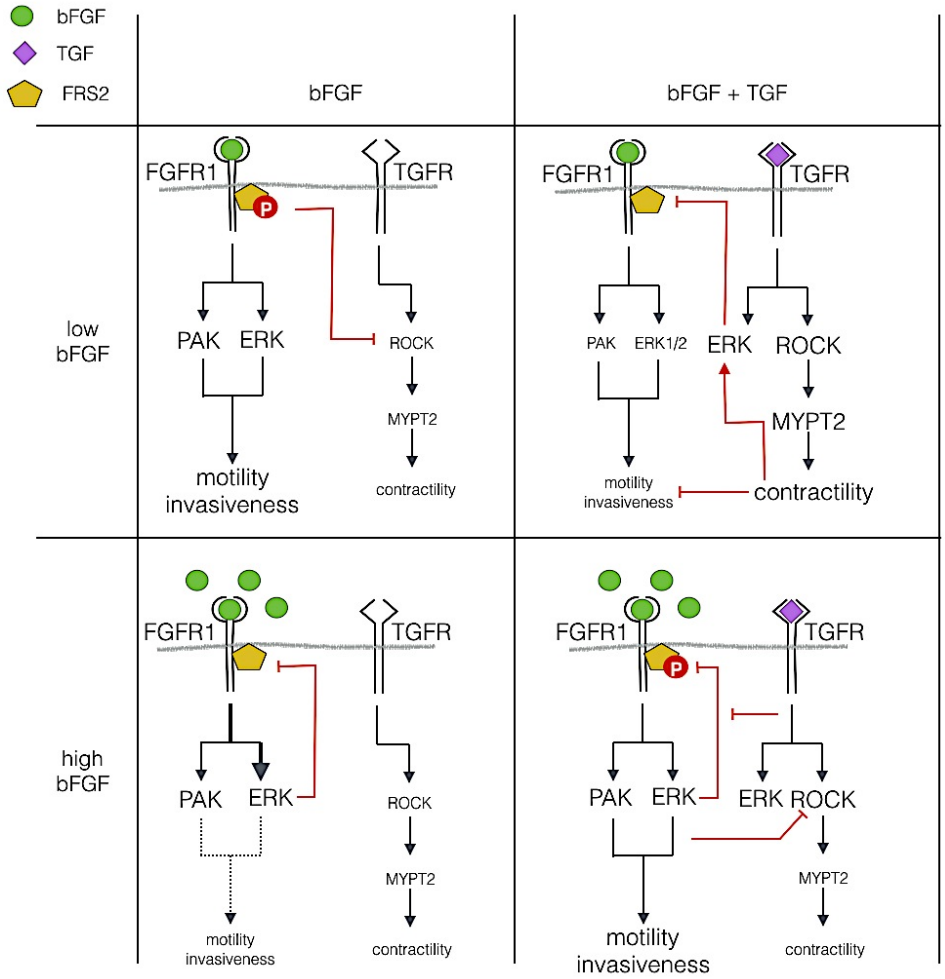
Suppl. Figure 6: bFGF is expressed in cerebellar slices and enriched near tumor spheroid (A) IFA anti-bFGF (red) and anti-Calbindin (green) in OCSC maintained in culture for 10 days. **(B)** Anti-bFGF (red) IFA in OCSC co-cultured for 5 d with DAOY LA-EGFP (green). Note strong anti-bFGF signal in and near tumor spheroid.



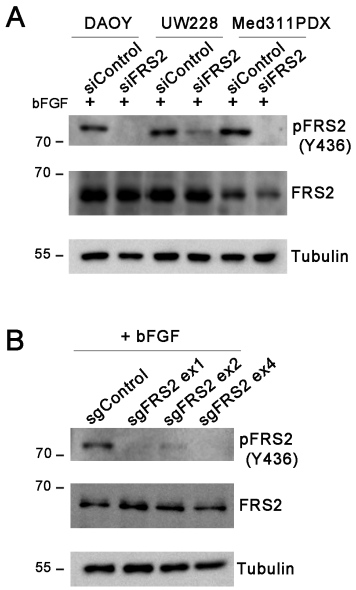
Suppl. Figure 7: (A) Dose response analysis as in Figure 6B in Med311PDX. **(B)** Line graphs of quantifications of SIAs using DAOY, UW228 and Med311PDX cells after treatment with bFGF -/+ TGF-β for 24 h (n=3, means ± SD).



Suppl. Figure 8: High concentration of bFGF impacts proliferation of DAOY but not Med311PDX cells. WST-1 assays in the presence of a range of bFGF concentrations using DAOY **(A)** or Med311PDX **(C)** in the absence **(A,C)** or presence **(B,D)** of 20 ng/ml TGF-β.



Suppl. Figure 9: Schematic representation of bFGF and TGF-β signaling pathway crosstalk at low and high concentrations of bFGF.



Suppl. Figure 10: (A) IB anti-pFRS2 of lysates of DAOY, UW228 and Med311PDX cells 48 h after transfection with siFRS2 or non-targeted siRNA (siControl). -/+ stimulation with 100 ng/ml bFGF for 10 minutes. **(B)** IB anti-pFRS2 of lysates of bFGF-stimulated DAOY sgControl, DAOY sgFRS2 ex1, DAOY sgFRS2 ex2 and DAOY sgFRS2 ex4 cells.

Manuscript 3: Tables

Table1: Dilutions of primary antibodies used

Antibody	Dilution	Application
bFGF	1:400	Immunohistochemistry
FGFR1	1:100	Immunohistochemistry
pERK1/2	1:750	Immunoblot
ERK1/2	1:1000	Immunoblot
pFRS2 (Y436)	1:750	Immunoblot
FRS2	1:1000	Immunoblot
FRS2	4µg of antibody for 500-1000µg/ml of protein	Immunoprecipitation
MyosinX	1:100	Immunofluorescence
ROCK1	1:1000	Immunoblot
ROCK2	1:1000	Immunoblot
pMYPT1 (Thr696)	1:500	Immunoblot
MYPT1	1:750	Immunoblot
RhoA	1:250	G-LISA
Rac1	1:20	G-LISA
Cdc42	1:50	G-LISA
Phospho-tyrosine (4G10)	1:1000	Immunoblot
GFAP	1:300	Immunofluorescence
Calbindin	1:1000	Immunofluorescence
pPKC	1:750	Immunoblot
Tubulin	1:1000	Immunoblot

Table 2: High and low levels of growth factors used for PB design

Growth Factor / Cytokine	High Level	Low Level
HGF	20ng/ml	0.039ng/ml
Netrin	200ng/ml	0.78ng/ml
EGF	30ng/ml	0.05ng/ml
TNF-α	25ng/ml	0.0975ng/ml
IGF	20ng/ml	0.039ng/ml
PDGF-B	20ng/ml	0.039ng/ml
TGF-β	20ng/ml	0.039ng/ml
IL-6	20ng/ml	0.039ng/ml
NGF	50ng/ml	0.195ng/ml
bFGF	100ng/ml	0.39ng/ml
PlGF-1	10ng/ml	0.078ng/ml

References:

Adachi, S., Yasuda, I., Nakashima, M., Yamauchi, T., Yoshioka, T., Okano, Y., Moriwaki, H., and Kozawa, O. (2011). Rho-kinase inhibitor upregulates migration by altering focal adhesion formation via the Akt pathway in colon cancer cells. Eur J Pharmacol *650*, 145-150.

Aref, D., Moffatt, C. J., Agnihotri, S., Ramaswamy, V., Dubuc, A. M., Northcott, P. A., Taylor, M. D., Perry, A., Olson, J. M., Eberhart, C. G., and Croul, S. E. (2013). Canonical TGF-beta Pathway Activity Is a Predictor of SHH-Driven Medulloblastoma Survival and Delineates Putative Precursors in Cerebellar Development. Brain Pathology *23*, 178-191.

Ashcroft, G. S., Yang, X., Glick, A. B., Weinstein, M., Letterio, J. J., Mizel, D. E., Anzano, M., Greenwell-Wild, T., Wahl, S. M., Deng, C. X., and Roberts, A. B. (1999). Mice lacking Smad3 show accelerated wound healing and an impaired local inflammatory response. Nature Cell Biology *1*, 260-266.

Bierie, B., and Moses, H. L. (2006). TGF beta: the molecular Jekyll and Hyde of cancer. Nature Reviews Cancer *6*, 506-520.

Bissell, M. J., and Radisky, D. (2001). Putting tumours in context. Nat Rev Cancer *1*, 46-54.

Bouffet, E., Bernard, J. L., Frappaz, D., Gentet, J. C., Roche, H., Tron, P., Carrie, C., Raybaud, C., Joannard, A., Lapras, C., and et al. (1992). M4 protocol for cerebellar medulloblastoma: supratentorial radiotherapy may not be avoided. Int J Radiat Oncol Biol Phys *24*, 79-85.

Cappellen, D., De Oliveira, C., Ricol, D., de Medina, S. G. D., Bourdin, J., Sastre-Garau, X., Chopin, D., Thiery, J. P., and Radvanyi, F. (1999). Frequent activating mutations of FGFR3 in human bladder and cervix carcinomas. Nature Genetics *23*, 18-20.

Corcoran, C., and O'Driscoll, L. (2015). Receptor Tyrosine Kinases and Drug Resistance: Development and Characterization of In Vitro Models of Resistance to RTK Inhibitors. Receptor Tyrosine Kinases: Methods and Protocols *1233*, 169-180.

da Silva, J. S., and Dotti, C. G. (2002). Breaking the neuronal sphere: regulation of the actin cytoskeleton in neuritogenesis. Nat Rev Neurosci *3*, 694-704.

Dent, E. W., Kwiatkowski, A. V., Mebane, L. M., Philippar, U., Barzik, M., Rubinson, D. A., Gupton, S., Van Veen, J. E., Furman, C., Zhang, J.*, et al.* (2007). Filopodia are required for cortical neurite initiation. Nat Cell Biol *9*, 1347-1359.

Derynck, R., and Zhang, Y. E. (2003). Smad-dependent and Smad-independent pathways in TGF-beta family signalling. Nature *425*, 577-584.

Dobolyi, A., Vincze, C., Pal, G., and Lovas, G. (2012). The Neuroprotective Functions of Transforming Growth Factor Beta Proteins. International Journal of Molecular Sciences *13*, 8219-8258.

Emmenegger, B. A., Hwang, E. I., Moore, C., Markant, S. L., Brun, S. N., Dutton, J. W., Read, T. A., Fogarty, M. P., Singh, A. R., Durden, D. L.*, et al.* (2013). Distinct roles for fibroblast growth factor signaling in cerebellar development and medulloblastoma. Oncogene *32*, 4181-4188.

Fiaschetti, G., Shalaby, T., Baumgartner, M., and Grotzer, M. A. (2014). Notch Ligands Jag1 and Jag2 Control Medulloblastoma Cell Survival and Represent Potential Prognostic Markers and Therapeutic Targets. Neuro-Oncology *16*, 74-74.

Fogarty, M. P., Emmenegger, B. A., Grasfeder, L. L., Oliver, T. G., and Wechsler-Reya, R. J. (2007). Fibroblast growth factor blocks Sonic hedgehog signaling in neuronal precursors and tumor cells. Proceedings of the National Academy of Sciences of the United States of America *104*, 2973-2978.

Freier, K., Schwaenen, C., Sticht, C., Flechtenmacher, C., Muhling, J., Hofele, C., Radlwimmer, B., Lichter, P., and Joos, S. (2007). Recurrent FGFR 1 amplification and high FGFR1 protein expression in oral squamous cell carcinoma (OSCC). Oral Oncology *43*, 60-66.

Friedl, P., and Wolf, K. (2009). Plasticity of cell migration: a multiscale tuning model. J Cell Biol, 11 - 19.

Fruhwald, M. C., and Plass, C. (2002). Metastatic medulloblastoma--therapeutic success through molecular target identification? Pharmacogenomics J *2*, 7-10.

Gate, D., Danielpour, M., Rodriguez, J., Kim, G. B., Levy, R., Bannykh, S., Breunig, J. J., Kaech, S. M., Flavell, R. A., and Town, T. (2014). T-cell TGF-beta signaling abrogation restricts medulloblastoma progression. Proceedings of the National Academy of Sciences of the United States of America *111*, E3458-E3466.

Gomez-Pinilla, F., Lee, J. W., and Cotman, C. W. (1994). Distribution of basic fibroblast growth factor in the developing rat brain. Neuroscience *61*, 911-923.

Gonzalez-Billault, C., Munoz-Llancao, P., Henriquez, D. R., Wojnacki, J., Conde, C., and Caceres, A. (2012). The role of small GTPases in neuronal morphogenesis and polarity. Cytoskeleton (Hoboken) *69*, 464-485.

Gotoh, N. (2008). Regulation of growth factor signaling by FRS2 family docking/scaffold adaptor proteins. Cancer Sci *99*, 1319-1325.

Guagnano, V., Furet, P., Spanka, C., Bordas, V., Le Douget, M., Stamm, C., Brueggen, J., Jensen, M. R., Schnell, C., Schmid, H.*, et al.* (2011). Discovery of 3-(2,6-dichloro-3,5-dimethoxy-phenyl)-1-{6-[4-(4-ethyl-piperazin-1-yl)-phenylamin o]-pyrimidin-4-yl}-1-methyl-urea (NVP-BGJ398), a potent and selective inhibitor of the fibroblast growth factor receptor family of receptor tyrosine kinase. Journal of medicinal chemistry *54*, 7066-7083.

Guo, X., and Wang, X. F. (2009). Signaling cross-talk between TGF-beta/BMP and other pathways. Cell Research *19*, 71-88.

Hatten, M. E., and Roussel, M. F. (2011). Development and cancer of the cerebellum. Trends Neurosci *34*, 134-142.

Heldin, C. H., Landstrom, M., and Moustakas, A. (2009). Mechanism of TGF-beta signaling to growth arrest, apoptosis, and epithelial-mesenchymal transition. Current opinion in cell biology *21*, 166-176.

Hetrick, B., Han, M. S., Helgeson, L. A., and Nolen, B. J. (2013). Small molecules CK-666 and CK-869 inhibit actin-related protein 2/3 complex by blocking an activating conformational change. Chem Biol *20*, 701-712.

Holland, E. C., and Varmus, H. E. (1998). Basic fibroblast growth factor induces cell migration and proliferation after glia-specific gene transfer in mice. Proc Natl Acad Sci U S A *95*, 1218-1223.

Hutley, L., Shurety, W., Newell, F., McGeary, R., Pelton, N., Grant, J., Herington, A., Cameron, D., Whitehead, J., and Prins, J. (2004). Fibroblast growth factor 1: a key regulator of human adipogenesis. Diabetes *53*, 3097-3106.

Jacquemet, G., Hamidi, H., and Ivaska, J. (2015). Filopodia in cell adhesion, 3D migration and cancer cell invasion. Current opinion in cell biology *36*, 23-31.

Jacquemier, J., Adelaide, J., Parc, P., Penaultllorca, F., Planche, J., Delapeyriere, O., and Birnbaum, D. (1994). Expression of the Fgfr1 Gene in Human Breast-Carcinoma Cells. International Journal of Cancer *59*, 373-378.

Keles, G. E., Berger, M. S., Srinivasan, J., Kolstoe, D. D., Bobola, M. S., and Silber, J. R. (1995). Establishment and characterization of four human medulloblastoma-derived cell lines. Oncology Research *7*, 493-503.

Kerber, M. L., and Cheney, R. E. (2011). Myosin-X: a MyTH-FERM myosin at the tips of filopodia. Journal of Cell Science *124*, 3733-3741.

Kiltie, A. E., Lashford, L. S., and Gattamaneni, H. R. (1997). Survival and late effects in medulloblastoma patients treated with craniospinal irradiation under three years old. Med Pediatr Oncol *28*, 348-354.

Korobova, F., and Svitkina, T. (2008). Arp2/3 complex is important for filopodia formation, growth cone motility, and neuritogenesis in neuronal cells. Molecular Biology of the Cell *19*, 1561-1574.

Krieglstein, K., Zheng, F., Unsicker, K., and Alzheimer, C. (2011). More than being protective: functional roles for TGF-beta/activin signaling pathways at central synapses. Trends in Neurosciences *34*, 421-429.

Kumar, K. S., Pillong, M., Kunze, J., Burghardt, I., Weller, M., Grotzer, M. A., Schneider, G., and Baumgartner, M. (2015). Computer-assisted quantification of motile and invasive capabilities of cancer cells. Scientific Reports *5*.

Kunii, K., Davis, L., Gorenstein, J., Hatch, H., Yashiro, M., Di, B. A., Elbi, C., and Lutterbach, B. (2008). FGFR2-amplified gastric cancer cell lines require FGFR2 and Erbb3 signaling for growth and survival (vol 68, pg 2340, 2008). Cancer Research *68*, 3549-3549.

Lai, M., Guo, Y., Ma, J., Yu, H., Zhao, D., Fan, W., Ju, X., Sheikh, M. A., Malik, Y. S., Xiong, W.*, et al.* (2015). Myosin X regulates neuronal radial migration through interacting with N-cadherin. Front Cell Neurosci *9*, 326.

Lu, P., Weaver, V. M., and Werb, Z. (2012). The extracellular matrix: a dynamic niche in cancer progression. J Cell Biol *196*, 395-406.

MacDonald, T. J., Brown, K. M., LaFleur, B., Peterson, K., Lawlor, C., Chen, Y., Packer, R. J., Cogen, P., and Stephan, D. A. (2001). Expression profiling of medulloblastoma: PDGFRA and the RAS/MAPK pathway as therapeutic targets for metastatic disease. Nat Genet *29*, 143-152.

Maddox, A. S., and Burridge, K. (2003). RhoA is required for cortical retraction and rigidity during mitotic cell rounding. Journal of Cell Biology *160*, 255-265.

Marino, S. (2005). Medulloblastoma: developmental mechanisms out of control. Trends Mol Med *11*, 17-22.

Massague, J. (2008). TGF beta in cancer. Cell *134*, 215-230.

Massague, J. (2012). TGF beta signalling in context. Nature Reviews Molecular Cell Biology *13*, 616-630.

Matsuo, I., and Kimura-Yoshida, C. (2013). Extracellular modulation of Fibroblast Growth Factor signaling through heparan sulfate proteoglycans in mammalian development. Current Opinion in Genetics & Development *23*, 399-407.

Murphy, M., Drago, J., and Bartlett, P. F. (1990). Fibroblast growth factor stimulates the proliferation and differentiation of neural precursor cells in vitro. J Neurosci Res *25*, 463-475.

Nobes, C. D., and Hall, A. (1999). Rho GTPases control polarity, protrusion, and adhesion during cell movement. Journal of Cell Biology *144*, 1235-1244.

Northcott, P. A., Dubuc, A. M., Pfister, S., and Taylor, M. D. (2012). Molecular subgroups of medulloblastoma. Expert Rev Neurother *12*, 871-884.

Reuss, B., and von Bohlen und Halbach, O. (2003). Fibroblast growth factors and their receptors in the central nervous system. Cell and Tissue Research *313*, 139-157.

Sahai, E., and Marshall, C. J. (2003). Differing modes of tumour cell invasion have distinct requirements for Rho/ROCK signalling and extracellular proteolysis. Nat Cell Biol *5*, 711-719.

Shirakihara, T., Horiguchi, K., Miyazawa, K., Ehata, S., Shibata, T., Morita, I., Miyazono, K., and Saitoh, M. (2011). TGF-beta regulates isoform switching of FGF receptors and epithelial-mesenchymal transition. EMBO J *30*, 783-795.

Snoussi, K., Mahfoudh, W., Bouaouina, N., Fekih, M., Khairi, H., Helal, A. N., and Chouchane, L. (2010). Combined effects of IL-8 and CXCR2 gene polymorphisms on breast cancer susceptibility and aggressiveness. BMC Cancer *10*, 283.

Suraneni, P., Rubinstein, B., Unruh, J. R., Durnin, M., Hanein, D., and Li, R. (2012). The Arp2/3 complex is required for lamellipodia extension and directional fibroblast cell migration. Journal of Cell Biology *197*, 239-251.

Thoma, C. R., Zimmermann, M., Agarkova, I., Kelm, J. M., and Krek, W. (2014). 3D cell culture systems modeling tumor growth determinants in cancer target discovery. Advanced Drug Delivery Reviews *69*, 29-41.

Thuault, S., Comunale, F., Hasna, J., Fortier, M., Planchon, D., Elarouci, N., De Reynies, A., Bodin, S., Blangy, A., and Gauthier-Rouviere, C. (2016). The RhoE/ROCK/ARHGAP25 signaling pathway controls cell invasion by inhibition of Rac activity. Mol Biol Cell *27*, 2653-2661.

Torka, R., Thuma, F., Herzog, V., and Kirfel, G. (2006). ROCK signaling mediates the adoption of different modes of migration and invasion in human mammary epithelial tumor cells. Exp Cell Res *312*, 3857-3871.

Turner, N., and Grose, R. (2010). Fibroblast growth factor signalling: from development to cancer. Nat Rev Cancer *10*, 116-129.

Vogelstein, B., Papadopoulos, N., Velculescu, V. E., Zhou, S. B., Diaz, L. A., and Kinzler, K. W. (2013). Cancer Genome Landscapes. Science *339*, 1546-1558.

Wells, A., Kassis, J., Solava, J., Turner, T., and Lauffenburger, D. A. (2002). Growth factor-induced cell motility in tumor invasion. Acta Oncol *41*, 124-130.

Wong, G. S., and Rustgi, A. K. (2013). Matricellular proteins: priming the tumour microenvironment for cancer development and metastasis. Br J Cancer *108*, 755-761.

Wu, X., Northcott, P. A., Dubuc, A., Dupuy, A. J., Shih, D. J., Witt, H., Croul, S., Bouffet, E., Fults, D. W., Eberhart, C. G.*, et al.* (2012). Clonal selection drives genetic divergence of metastatic medulloblastoma. Nature *482*, 529-533.

Yang, S., and Kim, H. M. (2014). ROCK inhibition activates MCF-7 cells. PLoS One *9*, e88489.

Zhang, Y. E. (2009). Non-Smad pathways in TGF-beta signaling. Cell Research *19*, 128-139.

Zhou, W. C., Feng, X. J., Wu, Y. J., Benge, J., Zhang, Z., and Chen, Z. J. (2009). FGF-receptor substrate 2 functions as a molecular sensor integrating external regulatory signals into the FGF pathway. Cell Research *19*, 1165-1177.

5. Discussion

- Cell motility models
- Tumour microenvironment in medulloblastoma
- Complexity and interrelations of growth factors signaling pathways promoting medulloblastoma cell motility
- FRS2: a potential anti-metastatic therapy target in MB
- A step further: Ex vivo and in vivo models to validate targets of medulloblastoma cell motility
- Future perspectives

5. Discussion

Dissecting the signaling pathways and evaluating various intervention strategies to target cell motility has been an exciting endeavor for the past 3 decades in the field of cancer. In medulloblastoma, leptomeningeal dissemination (LMD) occurs in up to 32% of patients, yet targeting cell motility pertinent to MB is still in its pre-infancy stages. This is because the intrinsic and extrinsic factors that tune the process of LMD are poorly understood. Preliminary insights into the intrinsic control of MB dissemination have come from the recent studies on expression profiling and clonal selection of MB. Expression profiling has identified 85 differentially regulated genes in metastatic and non-metastatic MB. These genes are correlated with de-regulated kinases, abnormal actin cytoskeleton regulation and resistance to chemotherapy in metastasizing MB [83]. Apart from the cell intrinsic factors that influence metastasis, tumour progression or regression is also predominantly dependent on the extrinsic factors in the highly dynamic tumour microenvironment [872]. However, these extrinsic factors that contribute to LMD have not yet been studied in detail in MB. Therefore, in this study, we have revealed the growth factor signaling pathways that influence MB cell motility and have also identified potential targets to intervene with LMD in MB.

One such potential target is the receptor tyrosine kinase c-Met. We demonstrated that activation of c-Met in MB cells via its ligand HGF promotes MB cell dissemination in 2D and 3D environments. Furthermore, we showed that HGF-induced motile and invasive behavior in MB requires MAP4K4, which control F-actin cytoskeleton dynamics in cellular protrusions necessary for motility and invasiveness. In addition to this novel HGF-induced-MAP4K4-dependent growth factor induced signaling circuit, there will be other growth factors released from the tumour microenvironment that can trigger MB cell dissemination. To explore this possibility, we developed an assay platform to quantify cancer cell dissemination in 2D and 3D environments in high-throughput. This platform, which named as Automated Cell Dissemination Counter (aCDc), consists of cell-based assays, imaging devices for acquisition and software solutions for the quantification of the imaging data. Using aCDc, we identified bFGF, HGF and EGF as the strongest promoters of MB cell dissemination. Furthermore, aCDc also revealed that the potent pro-migratory bFGF-signaling pathway is countered by an inhibitory circuitry induced by TGF-β. This antagonizing crosstalk between bFGF and TGF-β cascades converges at the level of FRS2, which renders it an attractive target for an anti-dissemination therapy approach in MB. We demonstrated the key function of FRS2 for brain tissue infiltration and uncovered its regulation at the molecular level. Our discovery of the paradigmatic cross-talk regulation of metastatic capability provides important insights in intrinsic and extrinsic control of tumor dissemination.

5.1 Cell motility models

Aberrant induction and maintenance of a migratory phenotype in MB could be caused by a plethora of extrinsic factors and molecular processes coupled to cellular morphodynamics.

Deeper insights into these processes and the systematic study of the underlying mechanisms require innovative, high-throughput cell motility models that enable multidimensional visualization and quantification of cell motility in space and time. Many 2D and 3D cell motility models have been developed to monitor motile / invasive cell behavior [873]. However, none of these models validated the motile behavior of MB cells. Therefore, to enable quantification of MB cell motility in 2D and 3D, we have assembled a package of three cell migration assays and combined them with automated imaging and computational image analysis. This program suite is referred to as automated Cell Dissemination counter (aCDc). Although, we have validated aCDc for MB, it can be applied to study the cell migration / invasion of virtually all solid tumours.

5.1.1 Challenges in 2D environment

2D migration assays are excellent tools that enable cell migration to be distinguished from cell growth and allow evaluating the effect of the drugs specifically on cell migration and not on cell growth. The vast majority of 2D migration assays provides an initial glimpse of cell's locomotion and is performed on 2D surfaces for the sake of convenience. Although performed for the sake of ease, the data from the 2D assays can be extrapolated to clinics. For example, a study with renal cancer cells from patients showed a 20-fold increase in cancer cell migration towards calcium as compared to non-transformed cells in 2D models and in the clinics [874]. Similarly, in MB we observed EGF as a potent pro-migratory factor in 2D models, 3D models and ex-vivo models, thus highlighting the potential targeting of EGF signaling pathways in MB and emphasizing the straightforward translation of findings from 2D in vitro models to the clinics.

The simple 'Scratch assay' is the most popular and most commonly used 2D migration assay. Numerous studies have been carried out using the 'Scratch assay' to assess the effects of growth factors, cytokines and chemokines in various human cancers. However, scrapping off an area of cells releases additional factors from the wounded areas, which may influence cell migration. In addition, the scratch is often uneven which has to be considered during quantification [462, 463]. Therefore to overcome the above limitations, we adopted Oris cell migration assay (Zone exclusion assay) as a superior alternative to the scratch assay to create homogenously sized cell-free zones without releasing factors from wounded or cell-free zones (Manuscript 2).

One limitation of 2D assays including the zone exclusion assay is that the invasive capability of the cells cannot be addressed adequately, even when adherent cells are embedded in 3D matrix. This is due to marked differences in cells adherent to a rigid surface as compared to fully embedded cells in terms of cell signaling, motility modes and plasticity of migration (and discussed and referenced in [875]), which are critical parameters for tumour cell dissemination in vivo as well. Furthermore, this rigid and planar 2D surface fails to mimic the in vivo cell behavior. Studies comparing gene expression of invading cells in spheroids and 2D cultures have reported significant differences in the genes related to cell survival, proliferation, differentiation and response to therapy, thus showing that spheroids more closely resemble in vivo tumours [488]. Hence, in addition to the zone exclusion assay, we

used 3D assays to explore steady state and induced motile MB cancer cell behavior and to identify growth factors relevant for matrix invasion and dissemination. Indeed, steady-state migration of MB cells is restricted in 3D and requires a stimulus, whereas MB cells on a stiff 2D substrate such as glass or plastic migrate spontaneously into cell free areas. This suggests that the molecular mechanisms underlying MB motility differ between 2D and 3D conditions.

5.1.2 Challenges in 3D environment

The constitution of the 3D environment has a significant effect on the cells' migratory properties. The natural substrate for most cancer cells is the 3D, complex and dynamic ECM. Migration in 3D ECM not only involves translocation of the cell body but also ECM remodeling and degradation of ECM by proteases. To include these crucial factors of 3D migration in vitro, most of the 3D invasion assays often include natural matrix components like collagen, laminin or hybrid matrices like matrigel. Of note, the composition and proportions of matrix components can be adjusted to impact cell behavior and better emulate the tumour microenvironment in vitro. The above phenomenon is observed in a study where the behavior of fibroblasts was assessed in four different matrices [876]. Similarly, matrix components also influence MB cell invasion. We have observed that DAOY and UW228 cells (SHH MB) massively infiltrate the collagen matrix while little or no infiltration is seen in matrigel. However, the infiltration of MB cells was increased when the stiffness of the matrigel matrix was reduced, thus indicating the influence of matrix stiffness on MB cell invasion. Matrix stiffness also governs the 3D invasion of prostate carcinoma cells along with cell-matrix adhesion and proteolysis. In contrast, the MB D cell lines (D341, D283 and D425) showed no infiltration in both collagen and matrigel. We anticipate that this could be due to the lack of certain cell-adhesion molecules on the surface of these cells, which we detected at lower levels or found absent.

MB is known to disseminate via the leptomeninges of the brain. Hence, it is important to use a relevant matrix protein in the 3D assays to assess MB cell dissemination in an in vivo-like environment. We have aptly used collagen as the matrix for our 3D assays as collagen I is highly expressed in the leptomeninges and in the ECM surrounding blood vessels in MB [877]. Our 3D cell motility models are versatile where variable parameters can be tested. These parameters include the composition of the matrix and the growth factor stimuli. Although, it is useful to have a model where variable parameters can be tested, little is known about the mechanisms by which these biophysical and mechano-chemical properties influence cancer cell migration in 3D in vitro models. Especially in matrices like collagen, the microstructures such as the fibril density, length and diameter will influence the cell invasion [878]. Therefore we recommend to maintain the matrix properties as a constant, which in turn will enable to vary and study the effects of growth factors and vice versa. Throughout this study, we retained the same concentration of collagen, which facilitated us to assess the effects of growth factors without any variability. Studies have reported that highly variable and non-reproducible results may arise if there are variations in the matrix properties especially in the case of anti-migratory drug screening [879]. This might explain some of the failures of the drugs in vivo.

Taken together, the invading cells' mode of migration and proteolysis of ECM is critically dependent on the microstructure of the 3D model. To circumvent these fluctuations, nowadays, more controllable synthetic biomimetic ECM analogs are being developed [880]. Despite these advancements and efforts to emulate the in vivo scenario, the influence of two or more stromal cells, blood flow and blood vessels on the invasion of cancer cells can never be recreated in the 3D in in vitro models. These types of studies require more advanced ex vivo or in vivo models.

5.1.3 Challenges in quantification of cell motility

One of the major challenges of cell motility models is the effective quantification of cell migration in 2D and 3D environments. Several assays have tackled to automate high-throughput quantification of cell motility in 2D. However, these assays either failed to fully automatize quantification or required special equipment, which might not be at disposal in many laboratories. For example, a freeware 'TScratch' allows simple automated image analysis and quantification of the scratch assay. Characteristically, the scratched wounds are uneven, which is not considered when quantified by TScratch [462, 463]. We have successfully used our validated zone exclusion assay as a platform to develop aZEcs. aZEcs precisely quantifies 2D cell migration in a fully automated way and takes into account pipetting artefacts and eliminates the deficits before quantification. This salient feature is not found in any other available software.

The actual extent of invasion in 3D cannot be accurately quantified. 3D invasion assays generates huge amounts of data and some of it are compromised during quantification, which necessitates the development of advanced automated systems [491]. Assays to automatically determine the dissemination range of cells migrating in 3D were not available, when we initiated the study, mostly because of the difficulties to efficiently measure the distance between origin and endpoint of migration of cells migrating detached from a solid substrate. To tackle this problem, we established the microbeads invasion and the spheroid cell invasion assays, both measure how far cells have disseminated from a defined reference point into a matrix. The reference points are the surface of the microbeads and the centre of the spheroid, respectively.

Based on our established high-throughput, microbead invasion assay and spheroid invasion assay, we have developed software aMDIcs and aSDIcs respectively, to automatically quantify the distance of dissemination of MB cells. Several quantification approaches have attempted to quantify disseminated cells from cell aggregates in an automatized way. In contrast to our approach, most of these platforms used the number of disseminated cells as a means to represent the extent of dissemination [881]. We believe that the distance of dissemination is a true representation of the extent of dissemination. This is observed in our experiments with IGF and bFGF. When DAOY cells are stimulated with IGF, a larger number of cells disseminated a shorter distance as compared to smaller number of cells disseminated a longer distance when stimulated with bFGF. Extrapolating this observation to the in vivo context, it can be easily perceived that invading cells, which disseminate longer

distance are likely capable of metastasizing more distantly. However, in the context of local infiltration, both the number of cells and the distance of dissemination as quantified by aMDIcs and aSDIcs can be used as a way to denote cell dissemination.

To our knowledge aCDc is by far the most versatile, easy to use, fully automatic and un-biased cell motility / invasion quantification platform. aCDc saves the original and the processed images as log files, which can always be traced back to confirm appropriate quantification. In most of the currently available semi-automated image-based cell migration quantifications, the quantification results depend on the quality of the images. Images of low quality may not be quantified using the available algorithms. To circumvent this setback, the individual software namely, aZECs, aMDIcs and aSDIcs come with an additional parameters file, which aids in accurately adjusting the software according to the quality of the image and in clear-cut quantification of poor quality images.

5.2 Tumour microenvironment in medulloblastoma

The tumour microenvironment of brain tumours is significantly different from solid tumours, but some aspects between solid tumours and CNS tumours remain similar. One such aspect is the influence of growth factors, which modulate the cell migration of tumour cells. Growth factors in the tumour microenvironment define the context for cell migration. In our study, we have successfully applied aCDc to study the effect of growth factors / cytokines on MB cells and to identify the downstream effectors of signaling pathways such as HGF, EGF and bFGF that induce cell invasiveness in MB cells. These effectors are attractive targets for an effective anti-metastatic therapy.

5.2.1 HGF signaling pathway

The tight regulation of HGF/c-Met signaling that is observed in developmental stages and regeneration is lost in a variety of human cancers at multiple levels. Deregulated c-Met activation can be caused by MET gene amplification, activation mutations and / or autocrine or paracrine mechanisms. We found that c-Met expression is up regulated in the SHH subgroup and in a subset of Group 3 and Group 4 MB tumours. A recent study reported that c-Met contributes to MB tumour progression by causing dissemination in a subset of recurrent SHH tumours [135]. Similarly, others and we have revealed that HGF promotes MB cell motility [882]. This suggests that blocking c-Met could be an excellent anti-dissemination therapy in MB. We evaluated the potential of two pharmacological inhibitors of c-Met, namely PHA665752 and ARQ197, to inhibit cell dissemination in c-Met positive MB cells. Both inhibitors were effective in blocking HGF-induced dissemination in SHH MB but ARQ197 was cytotoxic for non-c-Met expressing MB cells (Manuscript 1). A study, which evaluated the specificity of ARQ197, demonstrated that ARQ197 inhibited cell viability with similar potency in both c-Met-addicted and c-Met non-addicted tumours. As expected, other c-Met inhibitors like crizotinib and PHA665752 specifically suppressed the growth and dissemination of c-Met addicted tumours. Further investigations revealed that ARQ197 exhibits its anti-tumour activity in a manner independent of c-Met status as it inhibits microtubule polymerization in addition to inhibiting c-Met [883].

PHA665752 and ARQ197 are in phase I and phase II clinical trials respectively for non-small cell lung cancer [884, 885]. A phase II study with ARQ197 in these patients showed an improvement in progression free survivals and overall survival. In other clinical trails, treatment with ARQ197 inhibited the growth of hepatocellular and pancreatic carcinomas [886]. At present, there are no clinical trails evaluating c-Met inhibitors for the treatment of MB. Based on our data, we suggest that c-Met inhibitors may serve as an effective anti-metastatic treatment strategy in c-Met positive MB tumours.

Further analysis of the HGF-c-Met signaling pathway in MB using aCDc revealed that PI3K acts downstream of c-Met to promote MB cell dissemination (Manuscript 3). Currently, no clinical trials are evaluating PI3K inhibitors in MB. However, these inhibitors have been tested in patients with CNS tumours [887]. Therefore, in addition to c-Met inhibitors, PI3K inhibitors might also aid as good anti-dissemination therapy strategy for HGF-induced MB cell dissemination. Furthermore, we have identified that MAP4K4 is required for HGF-induced MB cell motility and revealed that MAP4K4 could be a putative anti-dissemination therapy target in MB. MAP4K4 is activated by various growth factors including HGF, PDGF, TNF-α and integrin activation [888, 889]. Hence, different receptor-mediated pathways presumably contribute to MAP4K4-dependent MB cell dissemination. Subsequently, MAP4K4 could act as a converging hub to divert different extracellular cues towards morphodynamic processes promoting motility and invasiveness. Thus, our findings argue for the development of novel MAPK4K inhibitors. However, the upstream activators and downstream effectors of MAP4K4 in MB need to be elucidated for the effective and safe use of MAP4K4 inhibitors in the clinics.

5.2.2 EGF signaling pathway

EGF along with its receptors have been of much attention for the molecular targeting of cancer therapeutics, owing to their abnormal expression in many epithelial tumours and their influence on growth, survival and migration in malignant states. Others and we have showed that EGF is a strong promoter of MB cell dissemination [890]. Predictably, blocking EGF signaling by blocking its receptor can be a new avenue to tackle MB cell dissemination. Ongoing clinical trials are testing the EGFR inhibitor (erlotinib) in combination with chemotherapy and radiotherapy in children with CNS tumours. Data from these trials show that erlotinib combined with temozolomide is well tolerated in pediatric patients. Although no objective response was observed, the disease remained stable in MB patients with this treatment regimen [891].

Our detailed analysis of the EGF signaling pathway demonstrated that inhibiting MEK/ERK partially impedes EGF-induced cell dissemination in MB. This indicated that EGF might signal via the classical MAPK-MEK/ERK cascade in MB. Numerous small molecules that inhibit different molecular targets, at the different levels of the MAPK pathway, have been developed. MEK inhibitors represent the first selective inhibitors of MAPK pathway activation to enter clinical trails. Several MEK inhibitors have been examined in early-phase clinical trials but these trials are not taken forward due to pronounced toxicity and failure to

improve survival. Similar to the PI3K inhibitors, MEK inhibitors are not being evaluated for MB but are evaluated for other CNS tumours [892]. Likewise, currently there are two ERK inhibitors in Phase I studies including MK8353, a clinical grade analog of SCH772984 and BVD-523. SCH722984 demonstrated a significant antitumour activity against BRAF mutant, NRAF mutant and wild-type melanoma [893]. In MB cells, SCH772984 was the best inhibitor against dissemination compared to all the other inhibitors we tested (Manuscript 2). Further investigations in larger cohorts of MB are required to translate our findings to the clinics. Multiple signal transduction pathways converge at the level of MEK/ERK and inhibition of MEK/ERK may block the activity of several signaling pathways at the same time. However, multi-signal pathway blockades may generate intolerable side effects that limit or attenuate any therapeutic benefits arising from these inhibitors.

5.2.3 FGF signaling pathway

Specific small molecule inhibitors against FGFRs and novel ways to perturb FGF signaling like FGF traps have initiated a number of clinical trials with FGFR inhibitors (detailed in chapter 1.8.8). The mechanism of action of selective FGFR inhibitors is analogues to the other receptor tyrosine kinase inhibitors (RTKi). Most of the selective FGFR inhibitors are in phase 1 or phase 2 trials and further trials are required to determine the cancer-cell resistance to FGFR inhibitors. However, as most of the RTK signaling cascades share a significant number of downstream effectors, it could be speculated that cancer cells can develop resistance to FGFR inhibitors as well. FGFRs are mutation-free in MB; hence it could be an attractive target. We have validated BGJ398, a potent FGFR1-3 selective small molecule RTKi as a strategy to target FGF signaling in MB. However, FGF signaling plays a variety of important roles in the developing brain including neurogenesis and differentiation, axon branching, neuro-protection and learning and memory (detailed in chapter 1.8.6), which has to be accounted for the successful translation of FGFR inhibitors for treatment in MB.

Additional studies on FGF signaling cascade using aCDc revealed that ERK and PAK act downstream of FGF signaling in MB. Of note, EGF and FGF signaling pathways signal via ERK, whose inhibition may block both the signaling cascades in MB. However, for successful translation of ERK inhibitors for the treatment in MB patients, the effect of simultaneous inhibition of EGF and FGF signaling in the brain has to be evaluated. In addition to ERK inhibitors, PAK inhibitors may also be used to attenuate FGF signaling in MB. Several broad-range kinase inhibitors have demonstrated potent PAK inhibition, but such non-selective compounds have limited utility in the clinics. A potent inhibitor of PAK1, PF-3758309 inhibits growth of many tumour cell lines and xenografts in transgenic mouse models. However, PF-3758309 did not advance beyond phase I trails due to undesirable pharmacological properties, which is conferred by the large size of PAK1 and high flexibility of the catalytic pocket [894].

5.2.4 TGF-β signaling pathway

The use of TGFR inhibitors for cancer therapy has been a long-standing debate due to the dual role of TGF-β. Nevertheless, a number of TGF-β inhibitors have entered clinical trails for

the treatment of a variety of human cancers (detailed in chapter 1.9.10). The dual role and context-dependent signaling of TGF-β is evident in MB. TGF-β is shown to interact synergistically with SHH pathway in pancreatic cancer [895]. Hence, as seen in the pancreatic cancer, TGF-β and SHH pathway might have to be blocked to improve clinical outcome in MB. However in the context of MB, Positive nuclear staining of SMAD3 and canonical activation of TGF-β signalling antagonizes SHH pathway and correlates with prolonged survival of MB patients [896]. In contrast, TGF-β is expressed in MB through secretion by regulatory T cells and abrogation of T cell TGF-β signalling mitigates MB progression [897]. These disparities in the effect of TGF-β in MB call for a caution when TGF-β inhibition is considered as a therapeutic strategy in MB.

TGF-β signals through ERK and ROCK cascades in MB. It is well known that ROCK and its targets are involved in regulating actin cytoskeleton dynamics and are responsible for cell migration. Therefore, the role of ROCK in tumour cell invasion and metastasis has been one of the most extensively studied mechanisms. Most studies have reported a positive role of ROCK activation in enhancing tumour cell invasion and metastasis either via direct effects on the tumour cell or via indirect effects on the tumour microenvironment. These studies claim that inhibiting ROCK by chemical inhibitors leads to decreased tumour cell invasion and metastasis [898]. For example, Y27632 decreased breast cancer cell invasion and migration in vitro and in vivo [899]. Similarly, Y27632 also decreased invasion and motility of Ca0V3 and SK0V-3 ovarian cancer cell lines [900].

Contrasting to these studies demonstrating beneficial effects of ROCK inhibition, we have shown the detrimental effects of ROCK inhibition in MB. Y27632 induced massive dissemination of MB cells in 2D and 3D environments. Similar to our findings, other studies have also demonstrated the unfavourable effects of ROCK inhibition. Y27632 treatment activated dormant MCF-7 breast cancer cells [901] and also increased the invasion of SW620 colon cancer cells [902]. The contradicting effects of ROCK inhibition on tumour cell invasion and metastasis can be related to the plasticity of cancer cells in their mode of migration and to the activation of other pro-migratory signalling pathways such as Rac GTPase dependent signalling [898]. As a result, the precise role of ROCK in different types of cancer is context dependent, which makes the use of ROCK inhibitors for certain cancers including MB debatable.

5.2.5 Tumour microenvironment signatures

Genomic analyses of large cohorts of MB now allow the classification of four molecular subgroups with defined molecular, functional and clinical characteristics. We propose a complementary method of MB subgroup characterization that is based on the growth factor sensitivity profiles of the tumours. Indeed, using aCDc, we were able to identify the distinct differences between established MB cell lines, PDX lines and primary MB cells. Therefore, we postulate that the high throughput capabilities of our platform combined with primary or PDX material permit efficient co-clinical testing of potential anti-metastatic drugs. Since our functional assay is based on cell dissemination triggered by growth factors, co-clinical assessment of patient-derived material may provide more biologically relevant information

as compared to the gene expression data. An example is Placental growth factor-1 (PlGF-1) signaling, which along with its receptor neuropilin 1 is expressed in the majority of human MB and specifically contributes to growth and spread of group 3 tumours. Our data in non-WNT /SHH cells and group3 PDX cells support the notion of specific, pro-tumourigenic impact of PlGF-1. However, PlGF-1 does not promote dissemination in SHH MB. These differences in growth factor sensitivities have to be considered during the development of treatment regimens for different subgroups of MB patients as different populations of patients have different tumour microenvironment signatures. The difference in tumour microenvironment signatures my also contribute to the resistance to targeted therapies as the tumour cells may no longer be dependent on targeted signaling pathway. For example in MB cells, we observed that aCDc also identified the specific response of UW228 cells to IL-6 stimulation. Hence, in this case, an ideal target would be an effector that orchestrates HGF, EGF, bFGF and IL-6 pathways. This implicitly proves that aCDc may be used to identify specific differences among the cell types, which could be exploited to effectively target the relevant pathways. This type of tumour microenvironment signatures is not only relevant for MB but also for other cancers. Although different tumour microenvironment parameters were tested using different analysis platforms, such stromal signatures predicted invasion, metastases, recurrence and prognosis in hepatocellular carcinoma and ductal carcinoma in situ [903, 904]. Further refinement will be needed and larger sample sizes are necessary to determine the precise tumour cell response to specific microenvironment signatures. Towards this end, aCDc may be used for the rapid diagnostic evaluation of tumour cell response to microenvironment parameters, which may enhance the clinical outcome in MB and other solid tumours.

5.3 Complexity and interrelations of growth factors signaling pathways promoting medulloblastoma cell motility

The growth factor defined context of cell migration can be influenced by more than one growth factor at the same time. Therefore, the presence or absence or combinations of two or more of certain growth factors and the resulting concentration gradients of the chemotactic stimuli can produce different cell migration outcomes. Although a handful of studies have assessed the simultaneous effects of two or more growth factors, none of the studies have been carried out in MB.

Our findings have revealed that combinations of growth factors in the tumour microenvironment can modulate the outcome of MB cell motility (Manuscript 3). The outcome of MB cell motility majorly depends on the following three aspects:

I. Crosstalk between growth factor signaling pathways present in the tumour microenvironment of MB
II. Concentration of the growth factors in the microenvironment of MB
III. Plasticity in the mode of cell motility

5.3.1 Crosstalk between growth factor signaling pathways

The phenomenon of crosstalk and convergence among various cell-signaling pathways is relatively old and it is implicated in developmental and metabolic processes. It is well documented that throughout the developmental processes, there is a balance of signaling between TGFs and FGFs, which determines the fate of bone development (chondrogenesis). Interestingly, in chondrocytes, the interaction between TGF-β and FGF pathways can either be positive or negative depending on the context. FGF signaling can antagonize canonical SMAD signaling while it can also have positive effects on TGF-β signaling via its non-ERK/MAPK downstream effectors [616, 618]. This crosstalk can be speculated in other developmental processes and our data confirms the presence of an antagonizing crosstalk between FGF and TGF-β signaling in the developing cerebellum in MB (Manuscript 3). Although, interrelations between FGF and TGF-β during the developmental stages have been a long-standing observation, the underlying mechanism was not known. We have now clarified the mechanism and have found that FGF and TGF-β antagonizes each other via ERK and ROCK and they converge at the level of FRS2, which acts as a molecular hub for these two pathways.

Apart from the crosstalk between TGF-β and FGF signaling, these signaling pathways can interact with other signaling pathways such as SHH, which are crucial for MB. bFGF was found to interfere with SHH signaling in neuronal precursor and tumour cells and to restrain tumour formation in a mouse model of SHH MB [905]. Thus, although bFGF treatment elicits anti-tumorigenic responses in MB cell lines, its dissemination promoting functions calls for caution when bFGF treatment is considered as therapeutic strategy. The context of TGF-β signalling dictates its tumour-permissive or tumour regressive implications in human cancers. TGF-β can act synergistically with SHH in pancreatic cancer [895], while in the brain environment TGF-b antagonizes SHH pathway in MB [896]. These finding have further complicated the already-challenging task of understanding and treating MB cell dissemination.

5.3.2 Responding to growth factor gradient in the tumour microenvironment

Growth factors and cytokines, the most abundant, non-cellular components of the tumour microenvironment are able to create gradients within the tumours and the surrounding stroma. Accordingly, the cancer cells within the tumour microenvironment are differentially exposed to these growth factor gradients. The physical properties and the chemical growth factor gradients in turn influence cancer cell behavior resulting in a multiple biological / phenotypical outcome from the within the same tumour microenvironment [906]. Based on these existing growth factor gradients, we assumed that there would also be bFGF gradients within the tumour microenvironment of MB. Our assumption is further actualized based on bFGF-positive cells within the tumour mass of MB, which will create bFGF gradients in the tumour. Predictably, these gradients will determine the biological outcome of MB cells. Similar type of concentration dependent outcomes are observed in EGFR signaling. Unlike, bFGF gradients, our data provide no evidence of TGF-β gradient in MB because any concentration between 20-200ng/ml of TGF-β confers the same outcome. Therefore, we

assumed TGF-β to be either present (in concentration > 20ng/ml) or absent in the tumour microenvironment of MB.

The described concentration gradients become more complex when another signaling pathway is activated within the gradient zone. This can be observed in the case of TGF-β and WNT signaling crosstalk where they reciprocally regulate their ligand production by establishing gradients [788]. Our findings reveal that activated TGF-β signaling in the vicinity of bFGF gradients can determine the migratory outcome of MB cells (Manuscript 3). We postulate that within the areas of the tumour infiltrated with bFGF positive cells, the concentration of bFGF is very high as compared to the areas without bFGF positive cells. Very high concentrations of bFGF when encountered by activated TGF-β provide a permissive migratory environment for MB cells while low concentrations of bFGF in combination with TGF-β are anti-migratory. Thus, TGF-β will be able to provide a migratory environment within tumor regions where very high concentrations of bFGF would otherwise restrict migration. Specifically, in the context of MB, TGF-β counters MB cell migration in the early stages of MB where bFGF positive cells are rarely present. However, as the tumour progresses, TGF-β promotes migration as more bFGF positive cells infiltrate the MB tumour mass, which create 'contexts' for TGF-β signaling (Figure 21). Therefore, our data exemplifies the notion that TGF-β elicits protective or tumour suppressive effects during the early stages of tumorigenesis, whereas in later stages it drives tumour progression [907]. This contextual signaling of TGF-β complicates targeting of TGF-β signaling in MB. Careful design of TGF-β targeted therapy, which can be adoptive according to the context of TGF-β in the patients' microenvironment, can be very challenging. Hence, we propose targeting FGF signaling as an effective alternative to therapeutically exploit this crosstalk in MB.

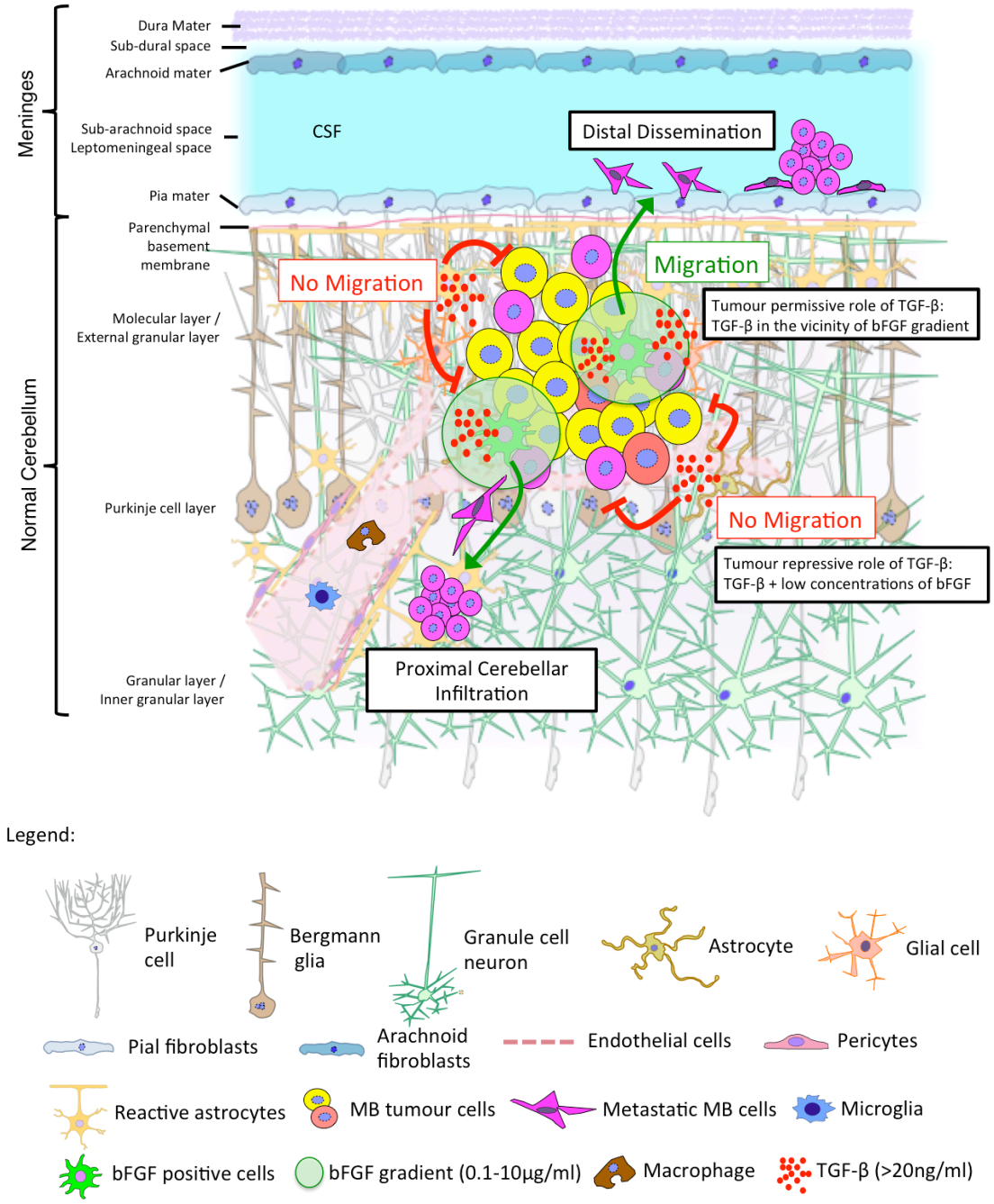


Figure 21: Context dependent signaling of TGF-β within bFGF gradients in medulloblastoma

In addition to the different concentrations of bFGF, the sulfation patterns and length of HS chains can also regulate FGF signaling [568]. In general, higher levels of sulfation of HS chains positively correlate with FGF pathway activation and formation of ternary complexes with FGFs and FGFRs. In addition, cleavage of HS core protein also influences FGF signaling by releasing FGFs that were sequestered at the cell-surface [570]. It is possible that different concentrations of bFGF coupled with HS and TGF-β can tune MB cells to a completely different biological outcome. As it is very complex to control three different variables at the same time in vitro, we did not address this possibility in our study. However, further studies on HS and bFGF may aid in the effective targeting on FGF signaling in MB.

5.3.3 Plasticity in the modes of cell motility

We revealed that bFGF-induced mesenchymal motility in the tumor cells is countered by an inhibitory circuitry induced by TGF-β via the activation of RhoA-ROCK, which is restricted by FGFR1-FRS2 signalling. Active RhoA/ROCK is required for diffuse cortical actin polymerization and cellular retraction [443]. Studies have shown that overexpression of constitutively active ROCK causes cortical contractions and cell rounding in cells, which originally adopted mesenchymal motility [407]. This is in line with our findings demonstrating that TGF-β-induced ROCK causes a rounded contractile phenotype in MB cells, which had previously adopted a bFGF-induced mesenchymal motility mode. Consistently, cancer cells have also been shown to switch modes of migration after ROCK inhibition; for instance, from rounded amoeboid type to elongated mesenchymal type in Y27632 treated gastric cancer cells [908]. We have elucidated this morphological switch upon ROCK inhibition also in MB.

It is well established that tumor cells exploit cellular plasticity and adopt different motility modes during tissue invasion. If mechanical or signaling pathways that stabilize the mesenchymal movement are weakened, cancer cells can convert towards amoeboid migration and vice versa. Therefore, drug combinations to simultaneously block several migratory signaling pathways may produce greater anti-metastatic effects. Combined inhibition of ROCK and Rac reduced mesenchymal motility of Y27632 treated gastric cancer cells [909]. Analogously, combined inhibition of ROCK and myotonic dystrophy kinase-related Cdc42-binding kinases (MRCK) inhibited migration and invasion of lung, breast, melanoma, and pancreatic cancer cells [910]. This strategy can also be applied to target MB cell dissemination. But, testing different drug combinations was outside the scope of our study.

5.4 FRS2: a potential anti-metastatic therapy target in MB

FRS2 is a unique target in MB because it prevents dissemination of MB cells in 2 ways: a) by inactivating bFGF signalling and b) by increasing contractility (mimicking the anti-migratory phenotype of TGF-β). We have dissected the two major pathways (FGF and TGFR) influencing FRS2 in medulloblastoma. It is well known that signaling crosstalk can influence targeted therapies. Given the complexities of signaling cross talk, the possibility of FRS2 being regulated by other MB-relevant signaling is not to be ignored. The modulation of FRS2 via other signaling cascades especially through EGF remain unanswered in MB. Future studies are required to explore these regulatory pathways of FRS2 to take forward FRS2 as a potential targeted intervention in MB. Moreover, the regulatory crosstalk of FRS2 will provide insights on choices to use a future FRS2 inhibitor as a monotherapy or as a combination therapy with other inhibitors targeting the regulatory pathways of FRS2.

RTK are prone to mutations in cancer and the use of RTKi for cancers are closely associated with the development of resistance [911]. Therefore, one way to target RTK cascade effectively is to target specific downstream effectors of the signaling cascade. Unlike RTK, FRS2 lack the characteristic kinase domain; therefore mechanistically targeting FRS2 is less

prone to resistance. In addition, FGF signaling plays a variety of important roles in the developing brain (detailed in chapter 1.8.5). Hence, as a means to spare the other functions of FGF signaling in the developing brain of the pediatric patients and to target specifically MB cell dissemination, we propose to target FRS2. FRS2 is an exclusive downstream effector of FGF signaling and it is the major responsible element for FGF-induced cell dissemination in MB. This confers FRS2 as an effective anti-dissemination therapy target in MB.

5.5 A step further: Ex vivo and in vivo models to validate targets of medulloblastoma cell motility

Molecularly targeted therapies are tested in the pre-clinical models before it being tested in clinical trials. In our study, we have validated FRS2 as an anti-dissemination therapy target using the ex-vivo organotypic cerebellar slice cultures (OCSCs) as it confers the following advantages. This is an excellent tool to visualize the local-proximal infiltration of MB cells in an environment with native brain architecture. OCSCs not only provide the brain's native architecture for the tumour cells but also the vascular structure of the brain, which mimics more closely the in vivo tumour microenvironment [503]. Though, the slices are devoid of any blood flow and the capillaries no longer function, it is probable that they maintain the factors and cells in the neurovascular unit. A major difficulty concerning the development and testing of inhibitors for brain tumours is to make the inhibitors BBB permeable. The brain slice cultures can aid in studying the BBB dynamics and its influence on drug availability in brain tumours by adapting the semi-permeable membrane technique for culturing the brain slices. In this technique, cells can be cultured in the lower compartment and the brain slices in the upper compartment. Pore sizes of the membrane will determine substrate / cell that can diffuse into the slices (or) if slices can be directly co-cultured with other cells, thus imitating the BBB. However, there is no functional ex-vivo BBB model to assess this phenomenon [501]. Moreover, most of anti-migratory drugs target not only motility but also influence cell viability and proliferation of both the tumour and normal cells. Hence, it is difficult to distinguish the true target and effect of the anti-migratory drugs in in vivo models. Validating the targets in brain slice cultures circumvented the above limitation and helped us to access the effects of anti-migratory drugs on neuronal cells. In our study, we were able to analyze the effects of FGF signaling in this apt ex-vivo system as the mouse cerebellar slices expressed inherent concentrations of bFGF, which is similar to human cerebellum. Nevertheless, the use of murine bFGF to resolve the effects of bFGF on human MB cells in our experiments is still a point of deliberation.

A wide range of mouse models have been developed from primary and metastatic tumours, including environmentally induced models, human tumour xenografts in immunocompromised mice and genetically engineered mice in MB. In most of the studies performed using in vivo models, anti-metastatic therapies are tested in the same way as other targeted therapies. While anti-metastatic therapies tested in in vivo models capture the complexity of the metastatic process in a living system, visualization of the individual steps like local infiltration is challenging and extracting quantitative mechanistic data is usually very difficult. In addition, in in vivo models, the standard measurement for efficacy of

potential anti-metastatic drugs involves shrinkage of tumour size / volume. Anti-migratory agents would require more specific end-point measurements like disease stabilization and infiltration-free survival. Careful design of end-points pertinent to migration and advanced intravital imaging techniques can thus overcome these limitations in validating the anti-metastatic therapy targets in in vivo models of MB.

5.6 Future perspectives

Currently there are no commercially available inhibitors for adaptor proteins including FRS2. Adaptor proteins can be effectively targeted only by means of disturbing its interaction with their respective receptors or other adaptor proteins. Adaptor proteins cannot be tagged for degradation as a means of targeting because there is a possibility that other adaptor proteins may compensate and keep the targeted signaling pathway functional. By nature, targeting protein-protein interactions are complex due to the lack of enzymatic activity, which can be relatively easily targeted. Knowing this, we have initiated the development of novel FRS2 inhibitors, which disrupt the interactions between the PTB domain of FRS2 and FGFR1. Downregulation of FRS2 and indirect inhibition of FRS2 in vitro and ex vivo have shown promising results (Manuscript 3). Prospective in vivo studies are needed to evaluate the effectiveness of FRS2 inhibition as anti-metastatic therapy strategy in MB.

Targeting cell motility alone might not be sufficient to eradicate MB; but it may contribute to its control by restricting local infiltration, by limiting further dissemination and by preventing the evolution towards a more aggressive phenotype. Apart from molecularly targeted therapies, there is growing interest in proton therapy as a potential replacement for photon therapy, while high dose chemotherapy and autologous stem cell rescue may improve therapeutic efficacies in MB. The prospect of treating MB with novel therapeutic strategies like immunotherapy, gene therapy, analogues stem cell rescue and proton therapy also need effective translation into the clinics via carefully executed clinical trials [912]. In conclusion, novel targeted therapies based on a better understanding of the biology of medulloblastomas coupled with chemotherapy and radiotherapy are pivotal in improving the existing conventional and novel non-conventional therapies in the treatment of this deadly disease.

6. Literature Cited

- References

6. Literature Cited

1. Siegel, R.L., K.D. Miller, and A. Jemal, *Cancer statistics, 2016.* CA Cancer J Clin, 2016. **66**(1): p. 7-30.
2. FSO, *Cancer in Switzerland - Situation and development from 1983 to 2007.* Federal Statistical Office, 2011.
3. Miller, K.D., et al., *Cancer treatment and survivorship statistics, 2016.* CA Cancer J Clin, 2016. **66**(4): p. 271-89.
4. Armstrong, G.T., Y. Yasui, and L.L. Robison, *Reduction in Late Mortality after Childhood Cancer.* N Engl J Med, 2016. **375**(3): p. 290-2.
5. Schindler, M., et al., *Childhood cancer survival in Switzerland (1976-2013): Time-trends and predictors.* Int J Cancer, 2016.
6. Downing, J.R., et al., *The Pediatric Cancer Genome Project.* Nat Genet, 2012. **44**(6): p. 619-22.
7. SCCR, *Swiss Childhood Cancer Registry* Annual Repost 2014/2015, 2014.
8. Downing, J.R., et al., *The Pediatric Cancer Genome Project.* Nature Genetics, 2012. **44**(6): p. 619-622.
9. Ward, E., et al., *Childhood and adolescent cancer statistics, 2014.* Ca-a Cancer Journal for Clinicians, 2014. **64**(2): p. 83-103.
10. Ward, E., et al., *Childhood and adolescent cancer statistics, 2014.* CA Cancer J Clin, 2014. **64**(2): p. 83-103.
11. Steliarova-Foucher, E., et al., *International Classification of Childhood Cancer, third edition.* Cancer, 2005. **103**(7): p. 1457-67.
12. Bleyer, A., et al., *The distinctive biology of cancer in adolescents and young adults.* Nat Rev Cancer, 2008. **8**(4): p. 288-98.
13. Smith, M.A., et al., *Outcomes for children and adolescents with cancer: challenges for the twenty-first century.* J Clin Oncol, 2010. **28**(15): p. 2625-34.
14. Pui, C.H., et al., *Challenging issues in pediatric oncology.* Nat Rev Clin Oncol, 2011. **8**(9): p. 540-9.
15. Pritchard-Jones, K., et al., *Sustaining innovation and improvement in the treatment of childhood cancer: lessons from high-income countries.* Lancet Oncol, 2013. **14**(3): p. e95-e103.
16. Hudson, M.M., et al., *Clinical ascertainment of health outcomes among adults treated for childhood cancer.* JAMA, 2013. **309**(22): p. 2371-81.
17. Hudson, M.M., et al., *Late mortality of long-term survivors of childhood cancer.* J Clin Oncol, 1997. **15**(6): p. 2205-13.
18. Meadows, A.T., et al., *Second neoplasms in survivors of childhood cancer: findings from the Childhood Cancer Survivor Study cohort.* J Clin Oncol, 2009. **27**(14): p. 2356-62.
19. Mertens, A.C., et al., *Late mortality experience in five-year survivors of childhood and adolescent cancer: the Childhood Cancer Survivor Study.* J Clin Oncol, 2001. **19**(13): p. 3163-72.
20. Tukenova, M., et al., *Radiation therapy and late mortality from second sarcoma, carcinoma, and hematological malignancies after a solid cancer in childhood.* Int J Radiat Oncol Biol Phys, 2011. **80**(2): p. 339-46.
21. Hanahan, D. and R.A. Weinberg, *The hallmarks of cancer.* Cell, 2000. **100**(1): p. 57-70.
22. Hanahan, D. and R.A. Weinberg, *Hallmarks of Cancer: The Next Generation.* Cell, 2011. **144**(5): p. 646-674.
23. Stratton, M.R., P.J. Campbell, and P.A. Futreal, *The cancer genome.* Nature, 2009. **458**(7239): p. 719-724.

24. Dippold, W., *Genetic Alterations during Colorectal-Tumor Development.* Zeitschrift Fur Gastroenterologie, 1989. **27**(1): p. 13-14.
25. Lawrence, M.S., et al., *Mutational heterogeneity in cancer and the search for new cancer-associated genes.* Nature, 2013. **499**(7457): p. 214-218.
26. Stratton, M., *Patterns of somatic mutation in human cancer genomes.* Chromosome Research, 2009. **17**: p. 16-16.
27. Vogelstein, B., et al., *Cancer Genome Landscapes.* Science, 2013. **339**(6127): p. 1546-1558.
28. Wu, G., et al., *Somatic histone H3 alterations in pediatric diffuse intrinsic pontine gliomas and non-brainstem glioblastomas.* Nature Genetics, 2012. **44**(3): p. 251-253.
29. Lee, R.S., et al., *A remarkably simple genome underlies highly malignant pediatric rhabdoid cancers.* Journal of Clinical Investigation, 2012. **122**(8): p. 2983-2988.
30. Mullighan, C.G., et al., *Genome-wide analysis of genetic alterations in acute lymphoblastic leukaemia.* Nature, 2007. **446**(7137): p. 758-764.
31. Tirode, F., et al., *Genomic Landscape of Ewing Sarcoma Defines an Aggressive Subtype with Co-Association of STAG2 and TP53 Mutations.* Cancer Discovery, 2014. **4**(11): p. 1342-1353.
32. Huether, R., et al., *The landscape of somatic mutations in epigenetic regulators across 1,000 paediatric cancer genomes.* Nature Communications, 2014. **5**.
33. Chen, X., et al., *Recurrent Somatic Structural Variations Contribute to Tumorigenesis in Pediatric Osteosarcoma.* Cell Reports, 2014. **7**(1): p. 104-112.
34. Northcott, P., et al., *Enhancer Hijacking Activates Gfi1 Family Oncogenes in Medulloblastoma.* Neuro-Oncology, 2014. **16**.
35. Cheung, N.K.V., et al., *Association of Age at Diagnosis and Genetic Mutations in Patients With Neuroblastoma.* Jama-Journal of the American Medical Association, 2012. **307**(10): p. 1062-1071.
36. Johnson, K.J., et al., *Childhood brain tumor epidemiology: a brain tumor epidemiology consortium review.* Cancer Epidemiol Biomarkers Prev, 2014. **23**(12): p. 2716-36.
37. Ostrom, Q.T., et al., *CBTRUS Statistical Report: Primary Brain and Central Nervous System Tumors Diagnosed in the United States in 2008-2012.* Neuro Oncol, 2015. **17 Suppl 4**: p. iv1-iv62.
38. Peris-Bonet, R., et al., *Childhood central nervous system tumours - incidence and survival in Europe (1978-1997): Report from Automated Childhood Cancer Information System Project.* European Journal of Cancer, 2006. **42**(13): p. 2064-2080.
39. Fleming, A.J. and S.N. Chi, *Brain Tumors in Children.* Current Problems in Pediatric and Adolescent Health Care, 2012. **42**(4): p. 80-103.
40. Crocetti, E., et al., *Epidemiology of glial and non-glial brain tumours in Europe.* Eur J Cancer, 2012. **48**(10): p. 1532-42.
41. Louis, D.N., et al., *The 2007 WHO classification of tumours of the central nervous system.* Acta Neuropathol, 2007. **114**(2): p. 97-109.
42. Nakazato, Y., *[The 4th Edition of WHO Classification of Tumours of the Central Nervous System published in 2007].* No Shinkei Geka, 2008. **36**(6): p. 473-91.
43. Albright, A.L., et al., *Correlation of neurosurgical subspecialization with outcomes in children with malignant brain tumors.* Neurosurgery, 2000. **47**(4): p. 879-885.
44. Gururangan, S., H.S. Friedman, and J.B. Powell, *Recent advances in the treatment of pediatric brain tumors.* Oncology-New York, 2004. **18**(13): p. 1649-1661.
45. DeLaney, T.F., *Clinical proton radiation therapy research at the Francis H. Burr Proton Therapy Center.* Technology in Cancer Research & Treatment, 2007. **6**(4): p. 61-66.

46. Merchant, T.E., I.F. Pollack, and J.S. Loeffler, *Brain Tumors Across the Age Spectrum: Biology, Therapy, and Late Effects.* Seminars in Radiation Oncology, 2010. **20**(1): p. 58-66.
47. Merchant, T.E., et al., *Proton versus photon radiotherapy for common pediatric brain tumors: Comparison of models of dose characteristics and their relationship to cognitive function.* Pediatric Blood & Cancer, 2008. **51**(1): p. 110-117.
48. Packer, R.J., et al., *Treatment of children with medulloblastomas with reduced-dose craniospinal radiation therapy and adjuvant chemotherapy: A children's cancer group study.* Journal of Clinical Oncology, 1999. **17**(7): p. 2127-2136.
49. Packer, R.J., et al., *Carboplatin and vincristine chemotherapy for children with newly diagnosed progressive low-grade gliomas.* Journal of Neurosurgery, 1997. **86**(5): p. 747-754.
50. Rood, B.R., T.J. MacDonald, and R.J. Packer, *Current treatment of medulloblastoma: Recent advances and future challenges.* Seminars in Oncology, 2004. **31**(5): p. 666-675.
51. Geyer, J.R., et al., *Multiagent chemotherapy and deferred radiotherapy in infants with malignant brain tumors: A report from the children's cancer group.* Journal of Clinical Oncology, 2005. **23**(30): p. 7621-7631.
52. Qaddoumi, I., I. Sultan, and A. Broniscer, *Pediatric low-grade gliomas and the need for new options for therapy Why and how?* Cancer Biology & Therapy, 2009. **8**(1): p. 4-10.
53. Partap, S. and P.G. Fisher, *Update on new treatments and developments in childhood brain tumors.* Current Opinion in Pediatrics, 2007. **19**(6): p. 670-674.
54. Mueller, S. and S. Chang, *Pediatric Brain Tumors: Current Treatment Strategies and Future Therapeutic Approaches.* Neurotherapeutics, 2009. **6**(3): p. 570-586.
55. Vredenburgh, J.J., et al., *Phase II trial of bevacizumab and irinotecan in recurrent malignant glioma.* Clinical Cancer Research, 2007. **13**(4): p. 1253-1259.
56. Broniscer, A., et al., *Phase I and Pharmacokinetic Studies of Erlotinib Administered Concurrently with Radiotherapy for Children, Adolescents, and Young Adults with High-Grade Glioma.* Clinical Cancer Research, 2009. **15**(2): p. 701-707.
57. Pollack, I.F., et al., *Phase I trial of imatinib in children with newly diagnosed brainstem and recurrent malignant gliomas: A Pediatric Brain Tumor Consortium report.* Neuro-Oncology, 2007. **9**(2): p. 145-160.
58. Turner, C.D., et al., *Medical, Psychological, Cognitive and Educational Late-Effects in Pediatric Low-Grade Glioma Survivors Treated With Surgery Only.* Pediatric Blood & Cancer, 2009. **53**(3): p. 417-423.
59. Hardy, K.K., et al., *Hydrocephalus as a possible additional contributor to cognitive outcome in survivors of pediatric medulloblastoma.* Psycho-Oncology, 2008. **17**(11): p. 1157-1161.
60. Palmer, S.L., et al., *Neurocognitive outcome 12 months following cerebellar mutism syndrome in pediatric patients with medulloblastoma.* Neuro-Oncology, 2010. **12**(12): p. 1311-1317.
61. Ullrich, N.J., et al., *Moyamoya following cranial irradiation for primary brain tumors in children.* Neurology, 2007. **68**(12): p. 932-938.
62. Armstrong, G.T., *Long-term survivors of childhood central nervous system malignancies: The experience of the Childhood Cancer Survivor Study.* European Journal of Paediatric Neurology, 2010. **14**(4): p. 298-303.
63. Turner, C.D., et al., *Late Effects of Therapy for Pediatric Brain Tumor Survivors.* Journal of Child Neurology, 2009. **24**(11): p. 1455-1463.
64. Hatten, M.E. and M.F. Roussel, *Development and cancer of the cerebellum.* Trends in Neurosciences, 2011. **34**(3): p. 134-142.

65. Rorke, L.B., *The Cerebellar Medulloblastoma and Its Relationship to Primitive Neuroectodermal Tumors.* Journal of Neuropathology and Experimental Neurology, 1983. **42**(1): p. 1-15.
66. Mueller, D.P., et al., *Mri-Spectrum of Medulloblastoma.* Clinical Imaging, 1992. **16**(4): p. 250-255.
67. Kumar, R., et al., *Medulloblastomas of the cerebellopontine angle.* Neurology India, 2001. **49**(4): p. 380-383.
68. Kumar, R., et al., *Uncommon presentation of medulloblastoma.* Childs Nervous System, 2001. **17**(9): p. 538-542.
69. Gilbertson, R.J. and D.W. Ellison, *The origins of medulloblastoma subtypes.* Annual Review of Pathology-Mechanisms of Disease, 2008. **3**: p. 341-365.
70. Valtz, N.L.M., et al., *An Embryonic Origin for Medulloblastoma.* New Biologist, 1991. **3**(4): p. 364-371.
71. Morales, D. and M.E. Hatten, *Molecular markers of neuronal progenitors in the embryonic cerebellar anlage.* Journal of Neuroscience, 2006. **26**(47): p. 12226-12236.
72. Marino, S., *Medulloblastoma: developmental mechanisms out of control.* Trends in Molecular Medicine, 2005. **11**(1): p. 17-22.
73. Shih, A.H. and E.C. Holland, *Developmental neurobiology and the origin of brain tumors.* Journal of Neuro-Oncology, 2004. **70**(2): p. 125-136.
74. Alder, J., et al., *Generation of cerebellar granule neurons in vivo by transplantation of BMP-treated neural progenitor cells.* Nature Neuroscience, 1999. **2**(6): p. 535-540.
75. Dahmane, N. and A.R.I. Altaba, *Sonic hedgehog regulates the growth and patterning of the cerebellum.* Development, 1999. **126**(14): p. 3089-3100.
76. Lewis, P.M., et al., *Sonic hedgehog signaling is required for expansion of granule neuron precursors and patterning of the mouse cerebellum.* Developmental Biology, 2004. **270**(2): p. 393-410.
77. Wechsler-Reya, R.J. and M.P. Scott, *Control of neuronal precursor proliferation in the cerebellum by sonic hedgehog.* Neuron, 1999. **22**(1): p. 103-114.
78. Yoon, K.J. and N. Gaiano, *Notch signaling in the mammalian central nervous system: insights from mouse mutants (vol 8, pg 709, 2005).* Nature Neuroscience, 2005. **8**(10): p. 1411-1411.
79. Rubinstein, L.J., *Extracranial Metastases in Cerebellar Medulloblastoma.* Journal of Pathology and Bacteriology, 1959. **78**(1): p. 187-&.
80. Fouladi, M., et al., *Comparison of CSF cytology and spinal magnetic resonance imaging in the detection of leptomeningeal disease in pediatric medulloblastoma or primitive neuroectodermal tumor.* Journal of Clinical Oncology, 1999. **17**(10): p. 3234-3237.
81. Packer, R.J., et al., *Leptomeningeal Dissemination of Primary Central Nervous-System Tumors of Childhood.* Annals of Neurology, 1985. **18**(2): p. 217-221.
82. Bai, A.H.C., et al., *MicroRNA-182 promotes leptomeningeal spread of non-sonic hedgehog-medulloblastoma.* Acta Neuropathologica, 2012. **123**(4): p. 529-538.
83. MacDonald, T.J., et al., *Expression profiling of medulloblastoma: PDGFRA and the RAS/MAPK pathway as therapeutic targets for metastatic disease (vol 29, pg 143, 2001).* Nature Genetics, 2003. **35**(3): p. 287-287.
84. Ramaswamy, S., et al., *A molecular signature of metastasis in primary solid tumors.* Nature Genetics, 2003. **33**(1): p. 49-54.
85. Wu, X., et al., *Clonal selection drives genetic divergence of metastatic medulloblastoma.* Nature, 2012. **482**(7386): p. 529-U254.
86. Fruhwald, M.C. and C. Plass, *Metastatic medulloblastoma--therapeutic success through molecular target identification?* Pharmacogenomics J, 2002. **2**(1): p. 7-10.

87. Ellison, D.W., *Childhood medulloblastoma: novel approaches to the classification of a heterogeneous disease.* Acta Neuropathologica, 2010. **120**(3): p. 305-316.
88. Louis, D.N., et al., *The 2007 WHO classification of tumours of the central nervous system (vol 114, pg 97, 2007).* Acta Neuropathologica, 2007. **114**(5): p. 547-547.
89. Lamont, J.M., et al., *Combined histopathological and molecular cytogenetic stratification of medulloblastoma patients.* Clinical Cancer Research, 2004. **10**(16): p. 5482-5493.
90. Ellison, D., *Classifying the medulloblastoma: insights from morphology and molecular genetics.* Neuropathology and Applied Neurobiology, 2002. **28**(4): p. 257-282.
91. McManamy, C.S., et al., *Nodule formation and desmoplasia in medulloblastomas - Defining the nodular/desmoplastic variant and its biological behavior.* Brain Pathology, 2007. **17**(2): p. 151-164.
92. Giangaspero, F., et al., *Large-Cell Medulloblastomas - a Distinct Variant with Highly Aggressive-Behavior.* American Journal of Surgical Pathology, 1992. **16**(7): p. 687-693.
93. McManamy, C.S., et al., *Morphophenotypic variation predicts clinical behavior in childhood non-desmoplastic medulloblastomas.* Journal of Neuropathology and Experimental Neurology, 2003. **62**(6): p. 627-632.
94. Brown, H.G., et al., *"Large cell/anaplastic" medulloblastomas: A Pediatric Oncology Group study.* Journal of Neuropathology and Experimental Neurology, 2000. **59**(10): p. 857-865.
95. Gulino, A., A. Arcella, and F. Giangaspero, *Pathological and molecular heterogeneity of medulloblastoma.* Current Opinion in Oncology, 2008. **20**(6): p. 668-675.
96. Pomeroy, S.L., et al., *Prediction of central nervous system embryonal tumour outcome based on gene expression.* Nature, 2002. **415**(6870): p. 436-442.
97. Taylor, M.D., et al., *Molecular subgroups of medulloblastoma: the current consensus.* Acta Neuropathologica, 2012. **123**(4): p. 465-472.
98. Northcott, P.A., et al., *Medulloblastoma Comprises Four Distinct Molecular Variants.* Journal of Clinical Oncology, 2011. **29**(11): p. 1408-1414.
99. Qualman, S.J., J. Bowen, and S.H. Erdman, *Molecular basis of the brain tumor-polyposis (Turcot) syndrome.* Pediatric and Developmental Pathology, 2003. **6**(6): p. 574-576.
100. Zurawel, R.H., et al., *Sporadic medulloblastomas contain oncogenic beta-catenin mutations.* Cancer Research, 1998. **58**(5): p. 896-899.
101. Schwalbe, E.C., et al., *Rapid diagnosis of medulloblastoma molecular subgroups.* Clin Cancer Res, 2011. **17**(7): p. 1883-94.
102. Kool, M., et al., *Molecular subgroups of medulloblastoma: an international meta-analysis of transcriptome, genetic aberrations, and clinical data of WNT, SHH, Group 3, and Group 4 medulloblastomas.* Acta Neuropathol, 2012. **123**(4): p. 473-84.
103. Ellison, D.W., et al., *Medulloblastoma: clinicopathological correlates of SHH, WNT, and non-SHH/WNT molecular subgroups.* Acta Neuropathol, 2011. **121**(3): p. 381-96.
104. Bale, S.J., R.T. Falk, and G.R. Rogers, *The basal cell nevus syndrome (Gorlin Syndrome): Genotype-phenotype correlation.* Journal of Investigative Dermatology, 1998. **110**(4): p. 502-502.
105. Brugieres, L., et al., *Incomplete penetrance of the predisposition to medulloblastoma associated with germ-line SUFU mutations.* Journal of Medical Genetics, 2010. **47**(2): p. 142-144.
106. Slade, I., et al., *Heterogeneity of familial medulloblastoma and contribution of germline PTCH1 and SUFU mutations to sporadic medulloblastoma.* Familial Cancer, 2011. **10**(2): p. 337-342.

107. Taylor, M.D., et al., *Mutations in SUFU predispose to medulloblastoma.* Nature Genetics, 2002. **31**(3): p. 306-310.
108. Northcott, P.A., et al., *Multiple Recurrent Genetic Events Converge on Control of Histone Lysine Methylation in Medulloblastoma.* Neuro-Oncology, 2009. **11**(6): p. 912-912.
109. Al-Halabi, H., et al., *Preponderance of sonic hedgehog pathway activation characterizes adult medulloblastoma.* Acta Neuropathologica, 2011. **121**(2): p. 229-239.
110. Ellison, D., et al., *Medulloblastoma - Clinicopathological Correlates of Shh, Wnt, and Non-Shh/Wnt Molecular Subgroups; a Study from the Pnet3 and Sjmb03 Study Groups.* Neuro-Oncology, 2011. **13**: p. I27-I28.
111. Garzia, L., et al., *Divergent Clonal Selection Dominates Medulloblastoma at Recurrence.* Neuro-Oncology, 2016. **18**: p. 119-119.
112. Ramaswamy, V., C. Nor, and M.D. Taylor, *p53 and Medulloblastoma (vol 6, a026278, 2016).* Cold Spring Harbor Perspectives in Medicine, 2016. **6**(4).
113. Adamson, D.C., et al., *OTX2 Is Critical for the Maintenance and Progression of Shh-Independent Medulloblastomas.* Cancer Research, 2010. **70**(1): p. 181-191.
114. de Haas, T., et al., *OTX1 and OTX2 expression correlates with the clinicopathologic classification of medulloblastomas.* Journal of Neuropathology and Experimental Neurology, 2006. **65**(2): p. 176-186.
115. Di, C.H., et al., *Identification of OTX2 as a medulloblastoma oncogene whose product can be targeted by all-trans retinoic acid.* Cancer Research, 2005. **65**(3): p. 919-924.
116. Kool, M., et al., *Molecular subgroups of medulloblastoma: an international meta-analysis of transcriptome, genetic aberrations, and clinical data of WNT, SHH, Group 3, and Group 4 medulloblastomas.* Acta Neuropathologica, 2012. **123**(4): p. 473-484.
117. Northcott, P., et al., *Integrative Genomics Identifies Actionable Targets for Therapy in Medulloblastoma Subgroups.* Neuro-Oncology, 2012. **14**: p. 87-87.
118. Ellison, D.W., et al., *Medulloblastoma: clinicopathological correlates of SHH, WNT, and non-SHH/WNT molecular subgroups.* Acta Neuropathologica, 2011. **121**(3): p. 381-396.
119. Ellison, D.W., *Childhood medulloblastoma: novel approaches to the classification of a heterogeneous disease.* Acta Neuropathol, 2010. **120**(3): p. 305-16.
120. Schwalbe, E.C., et al., *DNA methylation profiling of medulloblastoma allows robust subclassification and improved outcome prediction using formalin-fixed biopsies.* Acta Neuropathologica, 2013. **125**(3): p. 359-371.
121. Pizer, B.L. and S.C. Clifford, *The potential impact of tumour biology on improved clinical practice for medulloblastoma: progress towards biologically driven clinical trials.* British Journal of Neurosurgery, 2009. **23**(4): p. 364-375.
122. Simon, R.M., et al., *Using cross-validation to evaluate predictive accuracy of survival risk classifiers based on high-dimensional data.* Briefings in Bioinformatics, 2011. **12**(3): p. 203-214.
123. Ivanov, D.P., et al., *In vitro models of medulloblastoma: Choosing the right tool for the job.* Journal of Biotechnology, 2016. **236**: p. 10-25.
124. Heyer, J., et al., *Non-germline genetically engineered mouse models for translational cancer research.* Nature Reviews Cancer, 2010. **10**(7): p. 470-480.
125. Wetmore, C., D.E. Eberhart, and T. Curran, *The normal patched allele is expressed in medulloblastomas from mice with heterozygous germ-line mutation of patched.* Cancer Research, 2000. **60**(8): p. 2239-2246.
126. Hallahan, A.R., et al., *The SmoA1 mouse model reveals that notch signaling is critical for the growth and survival of sonic hedgehog-induced medulloblastomas.* Cancer Research, 2004. **64**(21): p. 7794-7800.

127. Marino, S., et al., *Induction of medulloblastomas in p53-null mutant mice by somatic inactivation of Rb in the external granular layer cells of the cerebellum.* Genes & Development, 2000. **14**(8): p. 994-1004.
128. Kongkham, P.N., et al., *The SFRP family of WNT inhibitors function as novel tumor suppressor genes epigenetically silenced in medulloblastoma.* Oncogene, 2010. **29**(20): p. 3017-3024.
129. Gibson, P., et al., *Subtypes of Medulloblastoma Have Distinct Developmental Origins.* Neuro-Oncology, 2011. **13**: p. I32-I33.
130. Smoll, N.R., *Relative survival of childhood and adult medulloblastomas and primitive neuroectodermal tumors (PNETs).* Cancer, 2012. **118**(5): p. 1313-1322.
131. McNeil, D.E., et al., *Incidence and trends in pediatric malignancies medulloblastoma/primitive neuroectodermal tumor: A SEER update.* Medical and Pediatric Oncology, 2002. **39**(3): p. 190-194.
132. Fisher, P.G., et al., *Medulloblastoma incidence has not changed over time: A study from the central brain tumor registry of the United States.* Neuro-Oncology, 2007. **9**(2): p. 199-199.
133. Kunschner, L.J., et al., *Survival and recurrence factors in adult medulloblastoma: The M.D. Anderson Cancer Center experience from 1978 to 1998.* Neuro-Oncology, 2001. **3**(3): p. 167-173.
134. Pui, C.H., et al., *Challenging issues in pediatric oncology.* Nature Reviews Clinical Oncology, 2011. **8**(9): p. 540-549.
135. Ramaswamy, V., M. Remke, and E. Bouffet, *Recurrence patterns across medulloblastoma subgroups: an integrated clinical and molecular analysis (vol 14, pg 1200, 2013).* Lancet Oncology, 2014. **15**(4): p. E154-E154.
136. Parsons, D.W., et al., *The Genetic Landscape of the Childhood Cancer Medulloblastoma.* Science, 2011. **331**(6016): p. 435-439.
137. Boca, S.M., et al., *Patient-oriented gene set analysis for cancer mutation data.* Genome Biology, 2010. **11**(11).
138. Northcott, P.A., J.T. Rutka, and M.D. Taylor, *Genomics of medulloblastoma: from Giemsa-banding to next-generation sequencing in 20 years.* Neurosurgical Focus, 2010. **28**(1).
139. Robinson, G., et al., *Novel mutations target distinct subgroups of medulloblastoma.* Nature, 2012. **488**(7409): p. 43-48.
140. Bouffet, E., et al., *M4 Protocol for Cerebellar Medulloblastoma - Supratentorial Radiotherapy May Not Be Avoided.* International Journal of Radiation Oncology Biology Physics, 1992. **24**(1): p. 79-85.
141. Berry, M.P., et al., *Radiation Treatment for Medulloblastoma - a 21-Year Review.* Journal of Neurosurgery, 1981. **55**(1): p. 43-51.
142. Kiltie, A.E., L.S. Lashford, and H.R. Gattamaneni, *Survival and late effects in medulloblastoma patients treated with craniospinal irradiation under three years old.* Medical and Pediatric Oncology, 1997. **28**(5): p. 348-354.
143. Leroy, R., et al., *Proton Therapy in Children: A Systematic Review of Clinical Effectiveness in 15 Pediatric Cancers.* International Journal of Radiation Oncology Biology Physics, 2016. **95**(1): p. 267-278.
144. Olsen, D.R., et al., *Proton therapy - A systematic review of clinical effectiveness.* Radiotherapy and Oncology, 2007. **83**(2): p. 123-132.
145. Mu, X.K., et al., *Does electron and proton therapy reduce the risk of radiation induced cancer after spinal irradiation for childhood medulloblastoma? A comparative treatment planning study.* Acta Oncologica, 2005. **44**(6): p. 554-562.
146. Smith, R.L., X. Shi, and E.J. Estlin, *Chemotherapy Dose-intensity and Survival for Childhood Medulloblastoma.* Anticancer Research, 2012. **32**(9): p. 3885-3892.

147. Packer, R.J., et al., *Improved Survival with the Use of Adjuvant Chemotherapy in the Treatment of Medulloblastoma.* Journal of Neurosurgery, 1991. **74**(3): p. 433-440.
148. Evans, A.E., et al., *The Treatment of Medulloblastoma - Results of a Prospective Randomized Trial of Radiation-Therapy with and without Ccnu, Vincristine, and Prednisone.* Journal of Neurosurgery, 1990. **72**(4): p. 572-582.
149. Ishida, T., et al., *Risk Adapted-Postoperative Chemotherapy and Optimization of a Dose of Cranial Irradiation for Childhood Medulloblastoma.* Annals of Oncology, 2014. **25**.
150. Pompe, R.S., et al., *Intraventricular methotrexate as part of primary therapy for children with infant and/or metastatic medulloblastoma: Feasibility, acute toxicity and evidence for efficacy Reply.* European Journal of Cancer, 2016. **56**: p. 181-182.
151. Grill, J., et al., *Phase II study of irinotecan in combination with temozolomide (TEMIRI) in children with recurrent or refractory medulloblastoma: a joint ITCC and SIOPE brain tumor study.* Neuro-Oncology, 2013. **15**(9): p. 1236-1243.
152. Kortmann, R.D., et al., *Postoperative neoadjuvant chemotherapy before radiotherapy as compared to immediate radiotherapy followed by maintenance chemotherapy in the treatment of medulloblastoma in childhood: Results of the German prospective randomized trial HIT '91.* International Journal of Radiation Oncology Biology Physics, 2000. **46**(2): p. 269-279.
153. Korah, M.P., et al., *Incidence, Risks, and Sequelae of Posterior Fossa Syndrome in Pediatric Medulloblastoma.* International Journal of Radiation Oncology Biology Physics, 2010. **77**(1): p. 106-112.
154. Anderson, D.M., et al., *Medical and neurocognitive late effects among survivors of childhood central nervous system tumors.* Cancer, 2001. **92**(10): p. 2709-2719.
155. Roman, D.D. and P.W. Sperduto, *Neuropsychological Effects of Cranial Radiation - Current Knowledge and Future-Directions.* International Journal of Radiation Oncology Biology Physics, 1995. **31**(4): p. 983-998.
156. Hoppehirsch, E., et al., *Medulloblastoma in Childhood - Progressive Intellectual Deterioration.* Childs Nervous System, 1990. **6**(2): p. 60-65.
157. Mostow, E.N., et al., *Quality-of-Life in Long-Term Survivors of Cns Tumors of Childhood and Adolescence.* Journal of Clinical Oncology, 1991. **9**(4): p. 592-599.
158. Olshan, J.S., et al., *The Effects of Adjuvant Chemotherapy on Growth in Children with Medulloblastoma.* Cancer, 1992. **70**(7): p. 2013-2017.
159. Pasqualini, T., et al., *Long-Term Endocrine Sequelae after Surgery, Radiotherapy, and Chemotherapy in Children with Medulloblastoma.* Cancer, 1987. **59**(4): p. 801-806.
160. Chin, D., et al., *Thyroid dysfunction as a late effect in survivors of pediatric medulloblastoma primitive neuroectodermal tumors - A comparison of hyperfractionated versus conventional radiotherapy.* Cancer, 1997. **80**(4): p. 798-804.
161. Duffner, P.K., et al., *Second malignancies in young children with primary brain tumors following treatment with prolonged postoperative chemotherapy and delayed irradiation: A Pediatric Oncology Group study.* Annals of Neurology, 1998. **44**(3): p. 313-316.
162. MacDonald, T.J., D. Aguilera, and R.C. Castellino, *The rationale for targeted therapies in medulloblastoma.* Neuro-Oncology, 2014. **16**(1): p. 9-20.
163. Samkari, A., J. White, and R. Packer, *SHH inhibitors for the treatment of medulloblastoma.* Expert Review of Neurotherapeutics, 2015. **15**(7): p. 763-770.
164. Rudin, C.M., et al., *Brief Report: Treatment of Medulloblastoma with Hedgehog Pathway Inhibitor GDC-0449.* New England Journal of Medicine, 2009. **361**(12): p. 1173-1178.

165. Geoerger, B., et al., *A phase I/II study of LDE225, a smoothened (Smo) antagonist, in pediatric patients with recurrent medulloblastoma (MB) or other solid tumors.* Journal of Clinical Oncology, 2012. **30**(15).
166. Yauch, R.L., et al., *Smoothened Mutation Confers Resistance to a Hedgehog Pathway Inhibitor in Medulloblastoma.* Science, 2009. **326**(5952): p. 572-574.
167. Fouladi, M., et al., *Phase I Trial of MK-0752 in Children With Refractory CNS Malignancies: A Pediatric Brain Tumor Consortium Study.* Journal of Clinical Oncology, 2011. **29**(26): p. 3529-3534.
168. Jakacki, R.I., et al., *Pediatric Phase I and Pharmacokinetic Study of Erlotinib Followed by the Combination of Erlotinib and Temozolomide: A Children's Oncology Group Phase I Consortium Study.* Journal of Clinical Oncology, 2008. **26**(30): p. 4921-4927.
169. Peyrl, A., et al., *Antiangiogenic metronomic therapy for children with recurrent embryonal brain tumors.* Pediatric Blood & Cancer, 2012. **59**(3): p. 511-517.
170. Santana, V.M., et al., *Phase I study of bevacizumab, sorafenib, and low-dose cyclophosphamide (CYC) in children and young adults with refractory solid tumors.* Journal of Clinical Oncology, 2011. **29**(15).
171. Milosevic, J., et al., *The cyclooxygenase-2 prostaglandin E2 pathway is expressed in childhood medulloblastoma providing novel therapeutic targets as indicated by promising response to Celecoxib-therapy in vitro, in vivo and pilot clinical experience.* Cancer Research, 2009. **69**.
172. Fouladi, M., et al., *Pediatric Phase I Trial and Pharmacokinetic Study of Vorinostat: A Children's Oncology Group Phase I Consortium Report.* Journal of Clinical Oncology, 2010. **28**(22): p. 3623-3629.
173. Hongeng, S., et al., *Treatment of leptomeningeal relapse of medulloblastoma with temozolomide.* Journal of Pediatric Hematology Oncology, 2002. **24**(7): p. 591-593.
174. Ruggiero, A., et al., *Phase I study of temozolomide combined with oral etoposide in children with recurrent or progressive medulloblastoma.* European Journal of Cancer, 2010. **46**(16): p. 2943-2949.
175. Fouladi, M., et al., *Carboplatin-based Primary Chemotherapy for Infants and Young Children With CNS Tumors.* Cancer, 2009. **115**(14): p. 3243-3253.
176. Gentet, J.C., et al., *Carboplatin and Vp-16 in Medulloblastoma - a Phase-Ii Study of the French-Society-of-Pediatric-Oncology (Sfop).* Medical and Pediatric Oncology, 1994. **23**(5): p. 422-427.
177. Silvani, A., et al., *Adult medulloblastoma: multiagent chemotherapy with cisplatinum and etoposide: a single institutional experience.* Journal of Neuro-Oncology, 2012. **106**(3): p. 595-600.
178. Hurwitz, C.A., et al., *Paclitaxel for the treatment of progressive or recurrent childhood brain tumors: A pediatric oncology phase II study.* Journal of Pediatric Hematology Oncology, 2001. **23**(5): p. 277-281.
179. Zwerdling, T., et al., *Phase II investigation of docetaxel in pediatric patients with recurrent solid tumors - A report from the Children's Oncology Group.* Cancer, 2006. **106**(8): p. 1821-1828.
180. Lu, P.F., V.M. Weaver, and Z. Werb, *The extracellular matrix: A dynamic niche in cancer progression.* Journal of Cell Biology, 2012. **196**(4): p. 395-406.
181. Bissell, M.J. and D. Radisky, *Putting tumours in context.* Nature Reviews Cancer, 2001. **1**(1): p. 46-54.
182. Liotta, L.A., et al., *Metastatic Potential Correlates with Enzymatic Degradation of Basement-Membrane Collagen.* Nature, 1980. **284**(5751): p. 67-68.
183. Egeblad, M., E.S. Nakasone, and Z. Werb, *Tumors as Organs: Complex Tissues that Interface with the Entire Organism.* Developmental Cell, 2010. **18**(6): p. 884-901.

184. Nguyen, D.X., P.D. Bos, and J. Massague, *Metastasis: from dissemination to organ-specific colonization.* Nature Reviews Cancer, 2009. **9**(4): p. 274-U65.
185. Bhowmick, N.A., E.G. Neilson, and H.L. Moses, *Stromal fibroblasts in cancer initiation and progression.* Nature, 2004. **432**(7015): p. 332-337.
186. Mueller, M.M. and N.E. Fusenig, *Friends or foes - Bipolar effects of the tumour stroma in cancer.* Nature Reviews Cancer, 2004. **4**(11): p. 839-849.
187. Sternlicht, M.D., et al., *The stromal proteinase MMP3/stromelysin-1 promotes mammary carcinogenesis.* Cell, 1999. **98**(2): p. 137-146.
188. Hadler-Olsen, E., J.O. Winberg, and L. Uhlin-Hansen, *Matrix metalloproteinases in cancer: their value as diagnostic and prognostic markers and therapeutic targets.* Tumor Biology, 2013. **34**(4): p. 2041-2051.
189. Wong, G.S. and A.K. Rustgi, *Matricellular proteins: priming the tumour microenvironment for cancer development and metastasis.* British Journal of Cancer, 2013. **108**(4): p. 755-761.
190. Frantz, C., K.M. Stewart, and V.M. Weaver, *The extracellular matrix at a glance.* Journal of Cell Science, 2010. **123**(24): p. 4195-4200.
191. Jarvelainen, H., et al., *Extracellular Matrix Molecules: Potential Targets in Pharmacotherapy.* Pharmacological Reviews, 2009. **61**(2): p. 198-223.
192. Schaefer, L. and R.M. Schaefer, *Proteoglycans: from structural compounds to signaling molecules.* Cell and Tissue Research, 2010. **339**(1): p. 237-246.
193. Rozario, T. and D.W. DeSimone, *The extracellular matrix in development and morphogenesis: A dynamic view.* Developmental Biology, 2010. **341**(1): p. 126-140.
194. Wise, S.G. and A.S. Weiss, *Tropoelastin.* International Journal of Biochemistry & Cell Biology, 2009. **41**(3): p. 494-497.
195. Smith, M.L., et al., *Force-induced unfolding of fibronectin in the extracellular matrix of living cells.* Plos Biology, 2007. **5**(10): p. 2243-2254.
196. Tsang, K.Y., et al., *The developmental roles of the extracellular matrix: beyond structure to regulation.* Cell and Tissue Research, 2010. **339**(1): p. 93-110.
197. Trebaul, A., E.K. Chan, and K.S. Midwood, *Regulation of fibroblast migration by tenascin-C.* Biochemical Society Transactions, 2007. **35**: p. 695-697.
198. Tucker, R.P. and R. Chiquet-Ehrismann, *The regulation of tenascin expression by tissue microenvironments.* Biochimica Et Biophysica Acta-Molecular Cell Research, 2009. **1793**(5): p. 888-892.
199. Friedl, P. and S. Alexander, *Cancer Invasion and the Microenvironment: Plasticity and Reciprocity.* Cell, 2011. **147**(5): p. 992-1009.
200. Hynes, R.O., *Integrins: Bidirectional, allosteric signaling machines.* Cell, 2002. **110**(6): p. 673-687.
201. Geiger, B., J.P. Spatz, and A.D. Bershadsky, *Environmental sensing through focal adhesions.* Nature Reviews Molecular Cell Biology, 2009. **10**(1): p. 21-33.
202. Grashoff, C., et al., *Measuring mechanical tension across vinculin reveals regulation of focal adhesion dynamics.* Nature, 2010. **466**(7303): p. 263-U143.
203. Ridley, A.J., et al., *Cell migration: Integrating signals from front to back.* Science, 2003. **302**(5651): p. 1704-1709.
204. Zoller, M., *CD44: can a cancer-initiating cell profit from an abundantly expressed molecule?* Nature Reviews Cancer, 2011. **11**(4): p. 254-267.
205. Couchman, J.R., *Transmembrane Signaling Proteoglycans.* Annual Review of Cell and Developmental Biology, Vol 26, 2010. **26**: p. 89-114.
206. Maaser, K., et al., *Functional hierarchy of simultaneously expressed adhesion receptors: Integrin alpha 2 beta 1 but not CD44 mediates MV3 melanoma cell migration and matrix reorganization within three-dimensional hyaluronan-*

containing collagen matrices. Molecular Biology of the Cell, 1999. **10**(10): p. 3067-3079.

207. Neuhaus, B., et al., *Migration inhibition of mammary epithelial cells by Syk is blocked in the presence of DDR1 receptors.* Cellular and Molecular Life Sciences, 2011. **68**(22): p. 3757-3770.
208. Vogel, W.F., R. Abdulhussein, and C.E. Ford, *Sensing extracellular matrix: An update on discoidin domain receptor function.* Cellular Signalling, 2006. **18**(8): p. 1108-1116.
209. Eswaramoorthy, R., et al., *DDR1 Regulates the Stabilization of Cell Surface E-Cadherin and E-Cadherin-Mediated Cell Aggregation.* Journal of Cellular Physiology, 2010. **224**(2): p. 387-397.
210. Ruiz, P.A. and G. Jarai, *Collagen I Induces Discoidin Domain Receptor (DDR) 1 Expression through DDR2 and a JAK2-ERK1/2-mediated Mechanism in Primary Human Lung Fibroblasts.* Journal of Biological Chemistry, 2011. **286**(15): p. 12912-12923.
211. Ilina, O. and P. Friedl, *Mechanisms of collective cell migration at a glance.* Journal of Cell Science, 2009. **122**(18): p. 3203-3208.
212. Berx, G. and F. van Roy, *Involvement of Members of the Cadherin Superfamily in Cancer.* Cold Spring Harbor Perspectives in Biology, 2009. **1**(6).
213. Harris, T.J.C. and U. Tepass, *Adherens junctions: from molecules to morphogenesis.* Nature Reviews Molecular Cell Biology, 2010. **11**(7): p. 502-514.
214. Friedl, P. and D. Gilmour, *Collective cell migration in morphogenesis, regeneration and cancer.* Nature Reviews Molecular Cell Biology, 2009. **10**(7): p. 445-457.
215. Zhang, X.J., D.T. Nie, and S. Chakrabarty, *Growth factors in tumor microenvironment.* Frontiers in Bioscience-Landmark, 2010. **15**: p. 151-165.
216. Elenbaas, B. and R.A. Weinberg, *Heterotypic signaling between epithelial tumor cells and fibroblasts in carcinoma formation.* Experimental Cell Research, 2001. **264**(1): p. 169-184.
217. Hughes, F.J., et al., *Effects of growth factors and cytokines on osteoblast differentiation.* Periodontology 2000, 2006. **41**: p. 48-72.
218. Lunt, S.J., N. Chaudary, and R.P. Hill, *The tumor microenvironment and metastatic disease.* Clinical & Experimental Metastasis, 2009. **26**(1): p. 19-34.
219. Wells, A., *Tumor invasion: Role of growth factor-induced cell motility.* Advances in Cancer Research, Vol 78, 2000. **78**: p. 31-101.
220. Seshacharyulu, P., et al., *Targeting the EGFR signaling pathway in cancer therapy.* Expert Opinion on Therapeutic Targets, 2012. **16**(1): p. 15-31.
221. Reddy, C.C., et al., *Engineering epidermal growth factor for enhanced mitogenic potency.* Nature Biotechnology, 1996. **14**(13): p. 1696-1699.
222. Chen, P., et al., *Epidermal Growth-Factor Receptor-Mediated Cell Motility - Phospholipase-C Activity Is Required, but Mitogen-Activated Protein-Kinase Activity Is Not Sufficient for Induced Cell-Movement.* Journal of Cell Biology, 1994. **127**(3): p. 847-857.
223. Price, J.T., et al., *Epidermal growth factor promotes MDA-MB-231 breast cancer cell migration through a phosphatidylinositol 3 '-kinase and phospholipase C-dependent mechanism.* Cancer Research, 1999. **59**(21): p. 5475-5478.
224. Santarpia, L., S.M. Lippman, and A.K. El-Naggar, *Targeting the MAPK-RAS-RAF signaling pathway in cancer therapy.* Expert Opinion on Therapeutic Targets, 2012. **16**(1): p. 103-119.
225. Lu, Z.M., et al., *Epidermal growth factor-induced tumor cell invasion and metastasis initiated by dephosphorylation and downregulation of focal adhesion kinase.* Molecular and Cellular Biology, 2001. **21**(12): p. 4016-4031.

226. Peruzzi, B. and D.P. Bottaro, *Targeting the c-Met signaling pathway in cancer.* Clinical Cancer Research, 2006. **12**(12): p. 3657-3660.
227. Kermorgant, S., et al., *Hepatocyte growth factor induces colonic cancer cell invasiveness via enhanced motility and protease overproduction. Evidence for PI3 kinase and PKC involvement.* Carcinogenesis, 2001. **22**(7): p. 1035-1042.
228. Mueller, K.L., et al., *Met and c-Src cooperate to compensate for loss of epidermal growth factor receptor kinase activity in breast cancer cells.* Cancer Research, 2008. **68**(9): p. 3314-3322.
229. Mamoune, A., et al., *DU145 human prostate carcinoma invasiveness is modulated by urokinase receptor (uPAR) downstream of epidermal growth factor receptor (EGFR) signaling.* Experimental Cell Research, 2004. **299**(1): p. 91-100.
230. Knudsen, B.S. and G.V. Woude, *Showering c-MET-dependent cancers with drugs.* Current Opinion in Genetics & Development, 2008. **18**(1): p. 87-96.
231. Metalli, D., et al., *The Insulin-Like Growth Factor Receptor I Promotes Motility and Invasion of Bladder Cancer Cells through Akt- and Mitogen-Activated Protein Kinase-Dependent Activation of Paxillin.* American Journal of Pathology, 2010. **176**(6): p. 2997-3006.
232. Hollier, B.G., et al., *Substrate-bound insulin-like growth factor (IGF)-I-IGF binding protein-vitronectin-stimulated breast cell migration is enhanced by coactivation of the phosphatidylinositide 3-Kinase/AKT pathway by alpha v-integrins and the IGF-I receptor.* Endocrinology, 2008. **149**(3): p. 1075-1090.
233. Zhang, D.L. and P. Brodt, *Type 1 insulin-like growth factor regulates MT1-MMP synthesis and tumor invasion via PI 3-kinase/Akt signaling.* Oncogene, 2003. **22**(7): p. 974-982.
234. Pickup, M., S. Novitskiy, and H.L. Moses, *The roles of TGF beta in the tumour microenvironment.* Nature Reviews Cancer, 2013. **13**(11): p. 788-799.
235. Papageorgis, P. and T. Stylianopoulos, *Role of TGF beta in regulation of the tumor microenvironment and drug delivery.* International Journal of Oncology, 2015. **46**(3): p. 933-943.
236. Giampieri, S., S. Pinner, and E. Sahai, *Intravital Imaging Illuminates Transforming Growth Factor beta Signaling Switches during Metastasis.* Cancer Research, 2010. **70**(9): p. 3435-3439.
237. Zilberberg, L., et al., *Specificity of latent TGF-beta binding protein (LTBP) incorporation into matrix: Role of fibrillins and fibronectin.* Journal of Cellular Physiology, 2012. **227**(12): p. 3828-3836.
238. Cheon, D.J., et al., *A Collagen-Remodeling Gene Signature Regulated by TGF-beta Signaling Is Associated with Metastasis and Poor Survival in Serous Ovarian Cancer.* Clinical Cancer Research, 2014. **20**(3): p. 711-723.
239. Balkwill, F. and A. Mantovani, *Cancer and Inflammation: Implications for Pharmacology and Therapeutics.* Clinical Pharmacology & Therapeutics, 2010. **87**(4): p. 401-406.
240. Germano, G., P. Allavena, and A. Mantovani, *Cytokines as a key component of cancer-related inflammation.* Cytokine, 2008. **43**(3): p. 374-379.
241. Sica, A., P. Allavena, and A. Mantovani, *Cancer related inflammation: The macrophage connection.* Cancer Letters, 2008. **267**(2): p. 204-215.
242. Mantovani, A., et al., *Cancer-related inflammation.* Nature, 2008. **454**(7203): p. 436-444.
243. Balkwill, F., *Tumour necrosis factor and cancer.* Nature Reviews Cancer, 2009. **9**(5): p. 361-371.

244. Montesano, R., et al., *Tumour necrosis factor alpha confers an invasive, transformed phenotype on mammary epithelial cells.* Journal of Cell Science, 2005. **118**(15): p. 3487-3500.
245. Hagemann, T., et al., *Macrophages induce invasiveness of epithelial cancer cells via NF-kappa B and JNK.* Journal of Immunology, 2005. **175**(2): p. 1197-1205.
246. Liang, M., P. Zhang, and J. Fu, *Up-regulation of LOX-1 expression by TNF-alpha promotes trans-endothelial migration of MDA-MB-231 breast cancer cells.* Cancer Letters, 2007. **258**(1): p. 31-37.
247. Kulbe, H., et al., *The inflammatory cytokine tumor necrosis factor-alpha regulates chemokine receptor expression on ovarian cancer cells.* Cancer Research, 2005. **65**(22): p. 10355-10362.
248. Karayiannakis, A.J., et al., *Serum levels of tumor necrosis factor-alpha and nutritional status in pancreatic cancer patients.* Anticancer Research, 2001. **21**(2B): p. 1355-1358.
249. Yoshida, N., et al., *Interleukin-6, tumour necrosis factor alpha and interleukin-1 beta in patients with renal cell carcinoma.* British Journal of Cancer, 2002. **86**(9): p. 1396-1400.
250. Bousserouel, S., et al., *Identification of gene expression profiles correlated to tumor progression in a preclinical model of colon carcinogenesis.* International Journal of Oncology, 2010. **36**(6): p. 1485-1490.
251. Beissert, S., et al., *Regulation of Tumor Necrosis Factor Gene-Expression in Colorectal Adenocarcinoma - Invivo Analysis by Insitu Hybridization.* Proceedings of the National Academy of Sciences of the United States of America, 1989. **86**(13): p. 5064-5068.
252. Pfitzenmaier, J., et al., *Elevation of cytokine levels in cachectic patients with prostate carcinoma.* Cancer, 2003. **97**(5): p. 1211-1216.
253. Vinader, V. and K. Afarinkia, *The emerging role of CXC chemokines and their receptors in cancer.* Future Medicinal Chemistry, 2012. **4**(7): p. 853-867.
254. Vandercappellen, J., J. Van Damme, and S. Struyf, *The role of CXC chemokines and their receptors in cancer.* Cancer Letters, 2008. **267**(2): p. 226-244.
255. Holland, J.D., et al., *Differential functional activation of chemokine receptor CXCR4 is mediated by G proteins in breast cancer cells.* Cancer Research, 2006. **66**(8): p. 4117-4124.
256. Zhao, M., et al., *Regulation of CXCR4-mediated nuclear translocation of extracellular signal-related kinases 1 and 2.* Molecular Pharmacology, 2006. **69**(1): p. 66-75.
257. Monterrubio, M., et al., *PI3K gamma activation by CXCL12 regulates tumor cell adhesion and invasion.* Biochemical and Biophysical Research Communications, 2009. **388**(2): p. 199-204.
258. Zagzag, D., et al., *Hypoxia-inducible factor 1 and VEGF upregulate CXCR4 in glioblastoma: implications for angiogenesis and glioma cell invasion.* Laboratory Investigation, 2006. **86**(12): p. 1221-1232.
259. Chinni, S.R., et al., *CXCL12/CXCR4 signaling activates Akt-1 and MMP-9 expression in prostate cancer cells: The role of bone microenvironment-associated CXCL12.* Prostate, 2006. **66**(1): p. 32-48.
260. Rahman, S., et al., *Novel hepatocyte growth factor (HGF) binding domains on fibronectin and vitronectin coordinate a distinct and amplified Met-integrin induced signalling pathway in endothelial cells.* Bmc Cell Biology, 2005. **6**.
261. Wijelath, E.S., et al., *Heparin-II domain of fibronectin is a vascular endothelial growth factor-binding domain - Enhancement of VEGF biological activity by a singular growth factor/matrix protein synergism.* Circulation Research, 2006. **99**(8): p. 853-860.

262. Rifkin, D.B., *Latent transforming growth factor-beta (TGF-beta) binding proteins: Orchestrators of TGF-beta availability.* Journal of Biological Chemistry, 2005. **280**(9): p. 7409-7412.
263. Wipff, P.J. and B. Hinz, *Integrins and the activation of latent transforming growth factor beta 1 - An intimate relationship.* European Journal of Cell Biology, 2008. **87**(8-9): p. 601-615.
264. Charles, N.A., et al., *The Brain Tumor Microenvironment.* Glia, 2011. **59**(8): p. 1169-1180.
265. Blouw, B., et al., *The hypoxic response of tumors is dependent on their microenvironment.* Cancer Cell, 2003. **4**(2): p. 133-146.
266. Lorger, M., et al., *Activation of tumor cell integrin alpha(v)beta(3) controls angiogenesis and metastatic growth in the brain.* Proceedings of the National Academy of Sciences of the United States of America, 2009. **106**(26): p. 10666-10671.
267. Guo, P., et al., *Vascular endothelial growth factor isoforms display distinct activities in promoting tumor angiogenesis at different anatomic sites.* Cancer Research, 2001. **61**(23): p. 8569-8577.
268. Deli, M.A., et al., *Permeability studies on in vitro blood-brain barrier models: Physiology, pathology, and pharmacology.* Cellular and Molecular Neurobiology, 2005. **25**(1): p. 59-127.
269. Fidler, I.J., et al., *The seed and soil hypothesis: vascularisation and brain metastases.* Lancet Oncology, 2002. **3**(1): p. 53-57.
270. Meyer, M.A., *Malignant gliomas in adults.* New England Journal of Medicine, 2008. **359**(17): p. 1850-1850.
271. Chao, H. and K.K. Hirschi, *Hemato-vascular origins of endothelial progenitor cells?* Microvascular Research, 2010. **79**(3): p. 169-173.
272. Dome, B., et al., *Circulating bone marrow-derived endothelial progenitor cells: Characterization, mobilization, and therapeutic considerations in malignant disease.* Cytometry Part A, 2008. **73A**(3): p. 186-193.
273. Baeriswyl, V. and G. Christofori, *The angiogenic switch in carcinogenesis.* Seminars in Cancer Biology, 2009. **19**(5): p. 329-337.
274. Hanahan, D. and J. Folkman, *Patterns and emerging mechanisms of the angiogenic switch during tumorigenesis.* Cell, 1996. **86**(3): p. 353-364.
275. IruelaArispe, M.L. and H.F. Dvorak, *Angiogenesis: A dynamic balance of stimulators and inhibitors.* Thrombosis and Haemostasis, 1997. **78**(1): p. 672-677.
276. Aicher, A., A.M. Zeiher, and S. Dimmeler, *Mobilizing endothelial progenitor cells.* Hypertension, 2005. **45**(3): p. 321-325.
277. Bertolini, F., et al., *The multifaceted circulating endothelial cell in cancer: towards marker and target identification.* Nature Reviews Cancer, 2006. **6**(11): p. 833-845.
278. Bergers, G. and S. Song, *The role of pericytes in blood-vessel formation and maintenance.* Neuro-Oncology, 2005. **7**(4): p. 452-464.
279. Hellstrom, M., et al., *Role of PDGF-B and PDGFR-beta in recruitment of vascular smooth muscle cells and pericytes during embryonic blood vessel formation in the mouse.* Development, 1999. **126**(14): p. 3047-3055.
280. Song, S., et al., *PDGFR beta(+) perivascular progenitor cells in tumours regulate pericyte differentiation and vascular survival.* Nature Cell Biology, 2005. **7**(9): p. 870-U16.
281. Carvalho, R.L.C., et al., *Defective paracrine signalling by TGF beta in yolk sac vasculature of endoglin mutant mice: a paradigm for hereditary haemorrhagic telangiectasia.* Development, 2004. **131**(24): p. 6237-6247.

282. Wurdak, H., et al., *Inactivation of TGF beta signaling in neural crest stem cells leads to multiple defects reminiscent of DiGeorge syndrome.* Genes & Development, 2005. **19**(5): p. 530-535.
283. Armulik, A., A. Abramsson, and C. Betsholtz, *Endothelial/pericyte interactions.* Circulation Research, 2005. **97**(6): p. 512-523.
284. Chekenya, M., et al., *The NG2 proteoglycan is expressed on angiogenic neovasculature by vascular pericytes and promotes tumour growth in CNS by sequestering angiostatin.* International Journal of Cancer, 2002: p. 336-336.
285. Davis, E.J., T.D. Foster, and W.E. Thomas, *Cellular-Forms and Functions of Brain Microglia.* Brain Research Bulletin, 1994. **34**(1): p. 73-78.
286. Guillemin, G.J. and B.J. Brew, *Microglia, macrophages, perivascular macrophages, and pericytes: a review of function and identification.* Journal of Leukocyte Biology, 2004. **75**(3): p. 388-397.
287. Morantz, R.A., et al., *Macrophages in Experimental and Human-Brain Tumors .2. Studies of the Macrophage Content of Human-Brain Tumors.* Journal of Neurosurgery, 1979. **50**(3): p. 305-311.
288. Morantz, R.A., et al., *Macrophages in Experimental and Human-Brain Tumors .1. Studies of the Macrophage Content of Experimental Rat-Brain Tumors of Varying Immunogenicity.* Journal of Neurosurgery, 1979. **50**(3): p. 298-304.
289. Klein, R. and W. Roggendorf, *Increased microglia proliferation separates pilocytic astrocytomas from diffuse astrocytomas: a double labeling study.* Acta Neuropathologica, 2001. **101**(3): p. 245-248.
290. Badie, B. and J. Schartner, *Role of microglia in glioma biology.* Microscopy Research and Technique, 2001. **54**(2): p. 106-113.
291. Leung, S.Y., et al., *Monocyte chemoattractant protein-1 expression and macrophage infiltration in gliomas.* Acta Neuropathologica, 1997. **93**(5): p. 518-527.
292. Yoshizato, K., et al., *Increased monocyte chemoattractant protein-1 expression by tumor necrosis factor-alpha can mediate macrophage infiltration in gliomas.* International Journal of Oncology, 1996. **8**(3): p. 493-497.
293. Kielian, T., N. van Rooijen, and W.F. Hickey, *MCP-1 expression in CNS-1 astrocytoma cells: implications for macrophage infiltration into tumors in vivo.* Journal of Neuro-Oncology, 2002. **56**(1): p. 1-12.
294. Liu, Y. and X.T. Cao, *The origin and function of tumor-associated macrophages.* Cellular & Molecular Immunology, 2015. **12**(1).
295. Markovic, D.S., et al., *Microglia stimulate the invasiveness of glioma cells by increasing the activity of metalloprotease-2.* Journal of Neuropathology and Experimental Neurology, 2005. **64**(9): p. 754-762.
296. Markovic, D.S., et al., *Gliomas induce and exploit microglial MT1-MMP expression for tumor expansion.* Proceedings of the National Academy of Sciences of the United States of America, 2009. **106**(30): p. 12530-12535.
297. Hoelzinger, D.B., T. Demuth, and M.E. Berens, *Autocrine factors that sustain glioma invasion and paracrine biology in the brain microenvironment.* Journal of the National Cancer Institute, 2007. **99**(21): p. 1583-1593.
298. Du, R., et al., *HIF1 alpha induces the recruitment of bone marrow-derived vascular modulatory cells to regulate tumor angiogenesis and invasion.* Cancer Cell, 2008. **13**(3): p. 206-220.
299. De Palma, M., et al., *Tie2 identifies a hematopoietic monocytes required for tumor lineage of proangiogenic vessel formation and a mesenchymal population of pericyte progenitors.* Cancer Cell, 2005. **8**(3): p. 211-226.
300. Galarneau, H., et al., *Increased glioma growth in mice depleted of macrophages.* Cancer Research, 2007. **67**(18): p. 8874-8881.

301. Flugel, A., et al., *Microglia only weakly present glioma antigen to cytotoxic T cells.* International Journal of Developmental Neuroscience, 1999. **17**(5-6): p. 547-556.
302. Huettner, C., et al., *Interleukin 10 is expressed in human gliomas in vivo and increases glioma cell proliferation and motility in vitro.* Anticancer Research, 1997. **17**(5A): p. 3217-3224.
303. Kiefer, R., et al., *In-Situ Detection of Transforming Growth-Factor-Beta Messenger-Rna in Experimental Rat Glioma and Reactive Glial-Cells.* Neuroscience Letters, 1994. **166**(2): p. 161-164.
304. Kuppner, M.C., et al., *Influence of Pge2-Modulating and Camp-Modulating Agents on Human Glioblastoma Cell Killing by Interleukin-2-Activated Lymphocytes.* Journal of Neurosurgery, 1990. **72**(4): p. 619-625.
305. Badie, B., et al., *Expression of Fas ligand by microglia: possible role in glioma immune evasion.* Journal of Neuroimmunology, 2001. **120**(1-2): p. 19-24.
306. Sofroniew, M.V., *Reactive astrocytes in neural repair and protection.* Neuroscientist, 2005. **11**(5): p. 400-407.
307. Sofroniew, M.V. and H.V. Vinters, *Astrocytes: biology and pathology.* Acta Neuropathologica, 2010. **119**(1): p. 7-35.
308. Zhang, M. and Y. Olsson, *Reactions of Astrocytes and Microglial Cells around Hematogenous Metastases of the Human Brain - Expression of Endothelin-Like Immunoreactivity in Reactive Astrocytes and Activation of Microglial Cells.* Journal of the Neurological Sciences, 1995. **134**(1-2): p. 26-32.
309. Marchetti, D., J. Li, and R. Shen, *Astrocytes contribute to the brain-metastatic specificity of melanoma cells by producing heparanase.* Cancer Research, 2000. **60**(17): p. 4767-4770.
310. Heinrich, P.C., et al., *Principles of interleukin (IL)-6-type cytokine signalling and its regulation.* Biochem J, 2003. **374**(Pt 1): p. 1-20.
311. Kostianovsky, A.M., et al., *Astrocytic regulation of human monocytic/microglial activation.* Journal of Immunology, 2008. **181**(8): p. 5425-5432.
312. Sameshima, T., et al., *Glioma cell extracellular matrix metalloproteinase inducer (EMMPRIN) (CD147) stimulates production of membrane-type matrix metalloproteinases and activated gelatinase A in co-cultures with brain-derived fibroblasts.* Cancer Letters, 2000. **157**(2): p. 177-184.
313. Lorger, M., *Tumor microenvironment in the brain.* International Journal of Molecular Medicine, 2011. **28**: p. S40-S40.
314. Charles, N., et al., *Perivascular Nitric Oxide Activates Notch Signaling and Promotes Stem-like Character in PDGF-Induced Glioma Cells.* Cell Stem Cell, 2010. **6**(2): p. 141-152.
315. Albini, A. and M.B. Sporn, *The tumour microenvironment as a target for chemoprevention.* Nature Reviews Cancer, 2007. **7**(2): p. 139-147.
316. Thun, M.J., S.J. Henley, and C. Patrono, *Nonsteroidal anti-inflammatory drugs as anticancer agents: Mechanistic, pharmacologic, and clinical issues.* Journal of the National Cancer Institute, 2002. **94**(4): p. 252-266.
317. Allegra, C.J., et al., *Bevacizumab in Stage II-III Colon Cancer: 5-Year Update of the National Surgical Adjuvant Breast and Bowel Project C-08 Trial.* Journal of Clinical Oncology, 2013. **31**(3): p. 359-364.
318. Shih, T. and C. Lindley, *Bevacizumab: An angiogenesis inhibitor for the treatment of solid malignancies.* Clinical Therapeutics, 2006. **28**(11): p. 1779-1802.
319. Garnock-Jones, K.P., G.M. Keating, and L.J. Scott, *Trastuzumab: a review of its use as adjuvant treatment in human epidermal growth factor receptor 2 (HER2)-positive early breast cancer (vol 70, pg 215, 2010).* Drugs, 2011. **71**(12): p. 1578-1578.

320. Garnock-Jones, K.P., G.M. Keating, and L.J. Scott, *Trastuzumab A Review of its Use as Adjuvant Treatment in Human Epidermal Growth Factor Receptor 2 (HER2)-Positive Early Breast Cancer.* Drugs, 2010. **70**(2): p. 215-239.
321. Lee, S.W., et al., *Blood-brain barrier interfaces and brain tumors.* Archives of Pharmacal Research, 2006. **29**(4): p. 265-275.
322. Nir, I., D. Levanon, and G. Iosilevsky, *Permeability of Blood-Vessels in Experimental Gliomas - Uptake of Tc-99m Glucoheptonate and Alteration in Blood-Brain-Barrier as Determined by Cyto-Chemistry and Electron-Microscopy.* Neurosurgery, 1989. **25**(4): p. 523-532.
323. Lockman, P.R., et al., *Heterogeneous Blood-Tumor Barrier Permeability Determines Drug Efficacy in Experimental Brain Metastases of Breast Cancer.* Clinical Cancer Research, 2010. **16**(23): p. 5664-5678.
324. Winkler, F., et al., *Imaging Glioma Cell Invasion In Vivo Reveals Mechanisms of Dissemination and Peritumoral Angiogenesis.* Glia, 2009. **57**(12): p. 1306-1315.
325. Jain, R.K., et al., *Angiogenesis in brain tumours.* Nature Reviews Neuroscience, 2007. **8**(8): p. 610-622.
326. Batchelor, T.T., et al., *AZD2171, a pan-VEGF receptor tyrosine kinase inhibitor, normalizes tumor vasculature and alleviates edema in glioblastoma patients.* Cancer Cell, 2007. **11**(1): p. 83-95.
327. Juan, J.Y., et al., *Noninvasive imaging of the functional effects of anti-VEGF therapy on tumor cell extravasation and regional blood volume in an experimental brain metastasis model.* Clinical & Experimental Metastasis, 2009. **26**(5): p. 403-414.
328. Kim, L.S., et al., *Vascular endothelial growth factor expression promotes the growth of breast cancer brain metastases in nude mice.* Clinical & Experimental Metastasis, 2004. **21**(2): p. 107-118.
329. Paez-Ribes, M., et al., *Antiangiogenic Therapy Elicits Malignant Progression of Tumors to Increased Local Invasion and Distant Metastasis.* Cancer Cell, 2009. **15**(3): p. 220-231.
330. Leenders, W.P.J., et al., *Antiangiogenic therapy of cerebral melanoma metastases results in sustained tumor progression via vessel co-option.* Clinical Cancer Research, 2004. **10**(18): p. 6222-6230.
331. De Palma, M., et al., *Tumor-targeted interferon-alpha delivery by Tie2-expressing monocytes inhibits tumor growth and metastasis.* Cancer Cell, 2008. **14**(4): p. 299-311.
332. Aboody, K.S., et al., *Neural stem cells display extensive tropism for pathology in adult brain: Evidence from intracranial gliomas (vol 97, pg 12846, 2000).* Proceedings of the National Academy of Sciences of the United States of America, 2001. **98**(2): p. 777-777.
333. Kim, S.K., et al., *Human neural stem cells target experimental intracranial medulloblastoma and deliver a therapeutic gene leading totumor regression.* Clinical Cancer Research, 2006. **12**(18): p. 5550-5556.
334. Balyasnikova, I.V., et al., *Mesenchymal Stem Cells Modified with a Single-Chain Antibody against EGFRvIII Successfully Inhibit the Growth of Human Xenograft Malignant Glioma.* Plos One, 2010. **5**(3).
335. Friedl, P. and E.B. Brocker, *The biology of cell locomotion within three-dimensional extracellular matrix.* Cellular and Molecular Life Sciences, 2000. **57**(1): p. 41-64.
336. Lauffenburger, D.A. and A.F. Horwitz, *Cell migration: A physically integrated molecular process.* Cell, 1996. **84**(3): p. 359-369.
337. Burridge, K. and M. ChrzanowskaWodnicka, *Focal adhesions, contractility, and signaling.* Annual Review of Cell and Developmental Biology, 1996. **12**: p. 463-518.

338. Huttenlocher, A., *Cell polarization mechanisms during directed cell migration.* Nature Cell Biology, 2005. **7**(4): p. 336-337.
339. Devreotes, P. and C. Janetopoulos, *Eukaryotic chemotaxis: Distinctions between directional sensing and polarization.* Journal of Biological Chemistry, 2003. **278**(23): p. 20445-20448.
340. Li, Z., et al., *Directional sensing requires G beta gamma-mediated PAK1 and PIX alpha-dependent activation of cdc42.* Cell, 2003. **114**(2): p. 215-227.
341. Welch, H.C.E., et al., *Phosphoinositide 3-kinase-dependent activation of Rac.* Febs Letters, 2003. **546**(1): p. 93-97.
342. Egeblad, M., M.G. Rasch, and V.M. Weaver, *Dynamic interplay between the collagen scaffold and tumor evolution.* Current Opinion in Cell Biology, 2010. **22**(5): p. 697-706.
343. Provenzano, P.P., et al., *Collagen reorganization at the tumor-stromal interface facilitates local invasion.* Bmc Medicine, 2006. **4**.
344. Levental, K.R., et al., *Matrix Crosslinking Forces Tumor Progression by Enhancing Integrin Signaling.* Cell, 2009. **139**(5): p. 891-906.
345. Pollard, T.D. and G.G. Borisy, *Cellular motility driven by assembly and disassembly of actin filaments (vol 112, pg 453, 2002).* Cell, 2003. **113**(4): p. 549-549.
346. Stupack, D.G., S.Y. Cho, and R.L. Klemke, *Molecular signaling mechanisms of cell migration and invasion.* Immunologic Research, 2000. **21**(2-3): p. 83-88.
347. Jacquemet, G., H. Hamidi, and J. Ivaska, *Filopodia in cell adhesion, 3D migration and cancer cell invasion.* Current Opinion in Cell Biology, 2015. **36**: p. 23-31.
348. Mattila, P.K. and P. Lappalainen, *Filopodia: molecular architecture and cellular functions.* Nature Reviews Molecular Cell Biology, 2008. **9**(6): p. 446-454.
349. Svitkina, T.M. and G.G. Borisy, *Arp2/3 complex and actin depolymerizing factor cofilin in dendritic organization and treadmilling of actin filament array in lamellipodia.* Journal of Cell Biology, 1999. **145**(5): p. 1009-1026.
350. Koestler, S.A., et al., *Differentially oriented populations of actin filaments generated in lamellipodia collaborate in pushing and pausing at the cell front.* Nature Cell Biology, 2008. **10**(3): p. 306-U33.
351. Hotulainen, P. and P. Lappalainen, *Stress fibers are generated by two distinct actin assembly mechanisms in motile cells.* Journal of Cell Biology, 2006. **173**(3): p. 383-394.
352. ChrzanowskaWodnicka, M. and K. Burridge, *Rho-stimulated contractility drives the formation of stress fibers and focal adhesions.* Journal of Cell Biology, 1996. **133**(6): p. 1403-1415.
353. Le Clainche, C. and M.F. Carlier, *Regulation of actin assembly associated with protrusion and adhesion in cell migration.* Physiological Reviews, 2008. **88**(2): p. 489-513.
354. Goldschmidtclermont, P.J., et al., *The Control of Actin Nucleotide Exchange by Thymosin-Beta-4 and Profilin - a Potential Regulatory Mechanism for Actin Polymerization in Cells.* Molecular Biology of the Cell, 1992. **3**(9): p. 1015-1024.
355. Yarmola, E.G. and M.R. Bubb, *Profilin: emerging concepts and lingering misconceptions.* Trends in Biochemical Sciences, 2006. **31**(4): p. 197-205.
356. Pollard, T.D. and J.A. Cooper, *Actin, a Central Player in Cell Shape and Movement.* Science, 2009. **326**(5957): p. 1208-1212.
357. Paul, A.S. and T.D. Pollard, *Review of the Mechanism of Processive Actin Filament Elongation by Formins.* Cell Motility and the Cytoskeleton, 2009. **66**(8): p. 606-617.
358. Chesarone, M.A., A.G. DuPage, and B.L. Goode, *Unleashing formins to remodel the actin and microtubule cytoskeletons.* Nature Reviews Molecular Cell Biology, 2010. **11**(1): p. 62-74.

359. Bear, J.E. and F.B. Gertler, *Ena/VASP: towards resolving a pointed controversy at the barbed end.* Journal of Cell Science, 2009. **122**(12): p. 1947-1953.
360. Bear, J.E., et al., *Antagonism between Ena/VASP proteins and actin filament capping regulates fibroblast motility.* Cell, 2002. **109**(4): p. 509-521.
361. Cooper, J.A. and D.A. Schafer, *Control of actin assembly and disassembly at filament ends.* Current Opinion in Cell Biology, 2000. **12**(1): p. 97-103.
362. Zigmond, S.H., *Beginning and ending an actin filament: Control at the barbed end.* Current Topics in Developmental Biology, Vol 63, 2004. **63**: p. 145-188.
363. Weaver, A.M., et al., *Integration of signals to the Arp2/3 complex.* Current Opinion in Cell Biology, 2003. **15**(1): p. 23-30.
364. LeClaire, L.L., et al., *The Nck-interacting kinase NIK increases Arp2/3 complex activity by phosphorylating the Arp2 subunit.* Journal of Cell Biology, 2015. **208**(2): p. 161-170.
365. Thrasher, A.J. and S.O. Burns, *WASP: a key immunological multitasker.* Nature Reviews Immunology, 2010. **10**(3): p. 182-192.
366. Hotulainen, P., et al., *Actin-depolymerizing factor and cofilin-1 play overlapping roles in promoting rapid F-actin depolymerization in mammalian nonmuscle cells.* Molecular Biology of the Cell, 2005. **16**(2): p. 649-664.
367. Carlier, M.F., et al., *Actin depolymerizing factor (ADF/cofilin) enhances the rate of filament turnover: Implication in actin-based motility.* Journal of Cell Biology, 1997. **136**(6): p. 1307-1322.
368. Zhao, X. and J.L. Guan, *Focal adhesion kinase and its signaling pathways in cell migration and angiogenesis.* Advanced Drug Delivery Reviews, 2011. **63**(8): p. 610-615.
369. Chishti, A.H., et al., *The FERM domain: a unique module involved in the linkage of cytoplasmic proteins to the membrane.* Trends in Biochemical Sciences, 1998. **23**(8): p. 281-282.
370. Girault, J.A., et al., *The N-termini of FAK and JAKs contain divergent band 4.1 domains.* Trends in Biochemical Sciences, 1999. **24**(2): p. 54-57.
371. Klemke, R.L., et al., *CAS/Crk coupling serves as a "molecular switch" for induction of cell migration.* Journal of Cell Biology, 1998. **140**(4): p. 961-972.
372. Cary, L.A., J.F. Chang, and J.L. Guan, *Stimulation of cell migration by overexpression of focal adhesion kinase and its association with Src and Fyn.* Journal of Cell Science, 1996. **109**: p. 1787-1794.
373. Cary, L.A., et al., *Identification of p130(Cas) as a mediator of focal adhesion kinase-promoted cell migration.* Journal of Cell Biology, 1998. **140**(1): p. 211-221.
374. Cho, S.Y. and R.L. Klemke, *Extracellular-regulated kinase activation and CAS/Crk coupling regulate cell migration and suppress apoptosis during invasion of the extracellular matrix.* Journal of Cell Biology, 2000. **149**(1): p. 223-236.
375. Turner, C.F., et al., *Paxillin LD4 motif binds PAK and PIX through a novel 95-kD ankyrin repeat, ARF-GAP protein: A role in cytoskeletal remodeling (vol 145, pg 851, 1999).* Journal of Cell Biology, 1999. **145**(7): p. 1523-1523.
376. West, K.A., et al., *The LD4 motif of paxillin regulates cell spreading and motility through an interaction with paxillin kinase linker (PKL).* Journal of Cell Biology, 2001. **154**(1): p. 161-176.
377. Lu, P.F., et al., *Extracellular Matrix Degradation and Remodeling in Development and Disease.* Cold Spring Harbor Perspectives in Biology, 2011. **3**(12).
378. Sternlicht, M.D. and Z. Werb, *How matrix metalloproteinases regulate cell behavior.* Annual Review of Cell and Developmental Biology, 2001. **17**: p. 463-516.

379. Page-McCaw, A., A.J. Ewald, and Z. Werb, *Matrix metalloproteinases and the regulation of tissue remodelling.* Nature Reviews Molecular Cell Biology, 2007. **8**(3): p. 221-233.
380. Cawston, T.E. and D.A. Young, *Proteinases involved in matrix turnover during cartilage and bone breakdown.* Cell and Tissue Research, 2010. **339**(1): p. 221-235.
381. Kessenbrock, K., V. Plaks, and Z. Werb, *Matrix Metalloproteinases: Regulators of the Tumor Microenvironment.* Cell, 2010. **141**(1): p. 52-67.
382. Apte, S.S., *A Disintegrin-like and Metalloprotease (Reprolysin-type) with Thrombospondin Type 1 Motif (ADAMTS) Superfamily: Functions and Mechanisms.* Journal of Biological Chemistry, 2009. **284**(46): p. 31493-31497.
383. Murphy, G., *The ADAMs: signalling scissors in the tumour microenvironment.* Nature Reviews Cancer, 2008. **8**(12): p. 929-941.
384. Smith, H.W. and C.J. Marshall, *Regulation of cell signalling by uPAR.* Nature Reviews Molecular Cell Biology, 2010. **11**(1): p. 23-36.
385. Rosen, S.D. and H. Lemjabbar-Alaoui, *Sulf-2: an extracellular modulator of cell signaling and a cancer target candidate.* Expert Opinion on Therapeutic Targets, 2010. **14**(9): p. 935-949.
386. Murrell, M., et al., *Forcing cells into shape: the mechanics of actomyosin contractility.* Nature Reviews Molecular Cell Biology, 2015. **16**(8): p. 486-498.
387. Dasanayake, N.L., P.J. Michalski, and A.E. Carlsson, *General Mechanism of Actomyosin Contractility.* Physical Review Letters, 2011. **107**(11).
388. Korn, E.D. and J.A. Hammer, *Myosins of Nonmuscle Cells.* Annual Review of Biophysics and Biophysical Chemistry, 1988. **17**: p. 23-45.
389. Thoresen, T., M. Lenz, and M.L. Gardel, *Reconstitution of Contractile Actomyosin Bundles.* Biophysical Journal, 2011. **100**(11): p. 2698-2705.
390. Bendix, P.M., et al., *A quantitative analysis of contractility in active cytoskeletal protein networks.* Biophysical Journal, 2008. **94**(8): p. 3126-3136.
391. Kirfel, G., et al., *Cell migration: mechanisms of rear detachment and the formation of migration tracks.* European Journal of Cell Biology, 2004. **83**(11): p. 717-724.
392. Perrin, B.J. and A. Huttenlocher, *Calpain effects contact mediated inhibition.* Molecular Biology of the Cell, 2002. **13**: p. 491A-491A.
393. Perrin, B.J. and A. Huttenlocher, *Calpain.* International Journal of Biochemistry & Cell Biology, 2002. **34**(7): p. 722-725.
394. Friedl, P., *Prespecification and plasticity: shifting mechanisms of cell migration.* Current Opinion in Cell Biology, 2004. **16**(1): p. 14-23.
395. Enterline, H.T. and D.R. Coman, *The Ameboid Motility of Human and Animal Neoplastic Cells.* Cancer, 1950. **3**(6): p. 1033-1038.
396. Simian, M., et al., *The interplay of matrix metalloproteinases, morphogens and growth factors is necessary for branching of mammary epithelial cells.* Development, 2001. **128**(16): p. 3117-3131.
397. Thiery, J.P., *Epithelial-mesenchymal transitions in tumour progression.* Nature Reviews Cancer, 2002. **2**(6): p. 442-454.
398. Wood, S., *Pathogenesis of Metastasis Formation Observed Invivo in the Rabbit Ear Chamber.* American Journal of Pathology, 1958. **34**(3): p. 576-576.
399. Grinnell, F., *Fibroblasts, Myofibroblasts, and Wound Contraction.* Journal of Cell Biology, 1994. **124**(4): p. 401-404.
400. Tamariz, E. and F. Grinnell, *Modulation of fibroblast morphology and adhesion during collagen matrix remodeling.* Molecular Biology of the Cell, 2002. **13**(11): p. 3915-3929.
401. Sameni, M., K. Moin, and B.F. Sloane, *Imaging proteolysis by living human breast cancer cells.* Neoplasia, 2000. **2**(6): p. 496-504.

402. d'Ortho, M.P., et al., *MT1-MMP on the cell surface causes focal degradation of gelatin films.* Febs Letters, 1998. **421**(2): p. 159-164.
403. Zamir, E., et al., *Dynamics and segregation of cell-matrix adhesions in cultured fibroblasts.* Nature Cell Biology, 2000. **2**(4): p. 191-196.
404. Petroll, W.M. and L.S. Ma, *Direct, dynamic assessment of cell-matrix interactions inside fibrillar collagen lattices.* Cell Motility and the Cytoskeleton, 2003. **55**(4): p. 254-264.
405. Keely, P.J., et al., *Cdc42 and Rac1 induce integrin-mediated cell motility and invasiveness through PI(3)K.* Nature, 1997. **390**(6660): p. 632-636.
406. Clark, E.A., et al., *Integrin-mediated signals regulated by members of the Rho family of GTPases.* Journal of Cell Biology, 1998. **142**(2): p. 573-586.
407. Nobes, C.D. and A. Hall, *Rho GTPases control polarity, protrusion, and adhesion during cell movement.* Journal of Cell Biology, 1999. **144**(6): p. 1235-1244.
408. Yumura, S., H. Mori, and Y. Fukui, *Localization of Actin and Myosin for the Study of Ameboid Movement in Dictyostelium Using Improved Immunofluorescence.* Journal of Cell Biology, 1984. **99**(3): p. 894-899.
409. Devreotes, P.N. and S.H. Zigmond, *Chemotaxis in Eukaryotic Cells - a Focus on Leukocytes and Dictyostelium.* Annual Review of Cell Biology, 1988. **4**: p. 649-686.
410. Fey, P., et al., *SadA, a novel adhesion receptor in Dictyostelium.* Journal of Cell Biology, 2002. **159**(6): p. 1109-1119.
411. Haston, W.S., J.M. Shields, and P.C. Wilkinson, *Lymphocyte Locomotion and Attachment on Two-Dimensional Surfaces and in 3-Dimensional Matrices.* Journal of Cell Biology, 1982. **92**(3): p. 747-752.
412. Verschueren, H., P. Debaetselier, and J. Bereiterhahn, *Dynamic Morphology of Metastatic Mouse T-Lymphoma Cells Invading through Monolayers of 10t1/2 Cells.* Cell Motility and the Cytoskeleton, 1991. **20**(3): p. 203-214.
413. Mandeville, J.T.H., M.A. Lawson, and F.R. Maxfield, *Dynamic imaging of neutrophil migration in three dimensions: Mechanical interactions between cells and matrix.* Journal of Leukocyte Biology, 1997. **61**(2): p. 188-200.
414. Wolf, K. and P. Friedl, *Extracellular matrix determinants of proteolytic and non-proteolytic cell migration.* Trends in Cell Biology, 2011. **21**(12): p. 736-744.
415. Friedl, P. and K. Wolf, *Proteolytic and non-proteolytic migration of tumour cells and leucocytes.* Proteases and the Regulation of Biological Processes, 2003. **70**: p. 277-285.
416. Worthylake, R.A., et al., *RhoA is required for monocyte tail retraction during transendothelial migration.* Journal of Cell Biology, 2001. **154**(1): p. 147-160.
417. Jacques, T.S., et al., *Neural precursor cell chain migration and division are regulated through different beta 1 integrins.* Development, 1998. **125**(16): p. 3167-3177.
418. El Fahime, E., et al., *In vivo migration of transplanted myoblasts requires matrix metalloproteinase activity.* Experimental Cell Research, 2000. **258**(2): p. 279-287.
419. Amano, M., et al., *Two cases of primary small cell carcinoma of the breast showing non-mass-like pattern on diagnostic imaging and histopathology.* Breast Cancer, 2015. **22**(4): p. 437-441.
420. Pitts, W.C., et al., *Carcinomas with Metaplasia and Sarcomas of the Breast.* American Journal of Clinical Pathology, 1991. **95**(5): p. 623-632.
421. Sood, A.K., et al., *Molecular determinants of ovarian cancer plasticity.* American Journal of Pathology, 2001. **158**(4): p. 1279-1288.
422. Seftor, E.A., et al., *Molecular determinants of human uveal melanoma invasion and metastasis.* Clinical & Experimental Metastasis, 2002. **19**(3): p. 233-246.
423. Petrie, R.J. and K.M. Yamada, *At the leading edge of three-dimensional cell migration.* Journal of Cell Science, 2012. **125**(24): p. 5917-5926.

424. Sixt, M., *Cell migration: Fibroblasts find a new way to get ahead.* Journal of Cell Biology, 2012. **197**(3): p. 346-348.
425. Petrie, R.J., et al., *Nonpolarized signaling reveals two distinct modes of 3D cell migration.* Journal of Cell Biology, 2012. **197**(3): p. 439-455.
426. Davidson, L.A. and R.E. Keller, *Neural tube closure in Xenopus laevis involves medial migration, directed protrusive activity, cell intercalation and convergent extension.* Development, 1999. **126**(20): p. 4547-4556.
427. Klinowska, T.C.M., et al., *Laminin and beta 1 integrins are crucial for normal mammary gland development in the mouse.* Developmental Biology, 1999. **215**(1): p. 13-32.
428. Vaughan, R.B. and J.P. Trinkaus, *Movements of Epithelial Cell Sheets in Vitro.* Journal of Cell Science, 1966. **1**(4): p. 407-&.
429. Nabeshima, K., et al., *Cohort migration of carcinoma cells: Differentiated colorectal carcinoma cells move as coherent cell clusters or sheets.* Histology and Histopathology, 1999. **14**(4): p. 1183-1197.
430. Klarlund, J.K., *Dual modes of motility at the leading edge of migrating epithelial cell sheets.* Proceedings of the National Academy of Sciences of the United States of America, 2012. **109**(39): p. 15799-15804.
431. Hegerfeldt, Y., et al., *Collective cell movement in primary melanoma explants: Plasticity of cell-cell interaction, ss 1-integrin function, and migration strategies.* Cancer Research, 2002. **62**(7): p. 2125-2130.
432. Nabeshima, K., et al., *Front-cell-specific expression of membrane-type 1 matrix metalloproteinase and gelatinase a during cohort migration of colon carcinoma cells induced by hepatocyte growth factor/scatter factor.* Cancer Research, 2000. **60**(13): p. 3364-3369.
433. Bradfield, J.W.B., *The Lives of Lesions - Chronology in Dermatopathology - Ackerman,Ab, Kagaz,A.* Lancet, 1984. **2**(8395): p. 138-139.
434. Friedl, P. and K. Wolf, *Tumour-cell invasion and migration: Diversity and escape mechanisms.* Nature Reviews Cancer, 2003. **3**(5): p. 362-374.
435. Hotary, K.B., et al., *Matrix metalloproteinases (MMPs) regulate fibrin-invasive activity via MT1-MMP-dependent and -independent processes.* Journal of Experimental Medicine, 2002. **195**(3): p. 295-308.
436. Pulyaeva, H., et al., *MT1-MMP correlates with MMP-2 activation potential seen after epithelial to mesenchymal transition in human breast carcinoma cells (vol 15, pg 117, 1997).* Clinical & Experimental Metastasis, 1997. **15**(3): p. 338-338.
437. Sakkab, D., et al., *Signaling of hepatocyte growth factor/scatter factor (HGF) to the small GTPase Rap1 via the large docking protein Gab1 and the adapter protein CRKL.* Journal of Biological Chemistry, 2000. **275**(15): p. 10772-10778.
438. Wolf, K., et al., *Compensation mechanism in tumor cell migration: mesenchymal-amoeboid transition after blocking of pericellular proteolysis.* Journal of Cell Biology, 2003. **160**(2): p. 267-277.
439. Overall, C.M. and C. Lopez-Otin, *Strategies for MMP inhibition in cancer: Innovations for the post-trial era.* Nature Reviews Cancer, 2002. **2**(9): p. 657-672.
440. Quirt, I., et al., *Phase II study of marimastat (BB-2516) in malignant melanoma - A clinical and tumor biopsy study of the National Cancer Institute of Canada Clinical Trials Group.* Investigational New Drugs, 2002. **20**(4): p. 431-437.
441. Ming, S.H., et al., *Matrix metalloproteinases-2,-9 and tissue inhibitor of metalloproteinase-1 in lung cancer invasion and metastasis.* Chinese Medical Journal, 2005. **118**(1): p. 69-72.
442. Bonomi, P., *Matrix metalloproteinases and matrix metalloproteinase inhibitors in lung cancer.* Seminars in Oncology, 2002. **29**(1): p. 78-86.

443. Maddox, A.S. and K. Burridge, *RhoA is required for cortical retraction and rigidity during mitotic cell rounding.* Journal of Cell Biology, 2003. **160**(2): p. 255-265.
444. Sahai, E. and C.J. Marshall, *Differing modes of tumour cell invasion have distinct requirements for Rho/ROCK signalling and extracellular proteolysis.* Nature Cell Biology, 2003. **5**(8): p. 711-719.
445. Fassler, R., et al., *Lack of Beta-1 Integrin Gene in Embryonic Stem-Cells Affects Morphology, Adhesion, and Migration but Not Integration into the Inner Cell Mass of Blastocysts.* Journal of Cell Biology, 1995. **128**(5): p. 979-988.
446. Fassler, R. and M. Meyer, *Consequences of Lack of Beta-1 Integrin Gene-Expression in Mice.* Genes & Development, 1995. **9**(15): p. 1896-1908.
447. Whittard, J.D. and S.K. Akiyama, *Activation of beta(1) integrins induces cell-cell adhesion.* Experimental Cell Research, 2001. **263**(1): p. 65-76.
448. Robinson, E.E., et al., *alpha 5 beta 1 integrin mediates strong tissue cohesion.* Journal of Cell Science, 2003. **116**(2): p. 377-386.
449. Wells, A., et al., *Targeting tumor cell motility as a strategy against invasion and metastasis.* Trends in Pharmacological Sciences, 2013. **34**(5): p. 283-289.
450. Palmer, T.D., et al., *Targeting tumor cell motility to prevent metastasis.* Advanced Drug Delivery Reviews, 2011. **63**(8): p. 568-581.
451. Waclaw, B., et al., *A spatial model predicts that dispersal and cell turnover limit intratumour heterogeneity.* Nature, 2015. **525**(7568): p. 261-+.
452. Ward, P.M. and L. Weiss, *Metachronous Seeding of Lymph-Node Metastases in Rats Bearing the Mt-100-Tc Mammary-Carcinoma - the Effect of Elective Lymph-Node Dissection.* Breast Cancer Research and Treatment, 1989. **14**(3): p. 315-320.
453. Klein, C.A., *Parallel progression of primary tumours and metastases.* Nature Reviews Cancer, 2009. **9**(4): p. 302-312.
454. Wu, Q., R. Dhir, and A. Wells, *Altered CXCR3 isoform expression regulates prostate cancer cell migration and invasion.* Molecular Cancer, 2012. **11**.
455. Kharait, S., et al., *Decision tree modeling predicts effects of inhibiting contractility signaling on cell motility.* Bmc Systems Biology, 2007. **1**.
456. Cristofanilli, M., *Circulating tumor cells, disease progression, and survival in metastatic breast cancer.* Seminars in Oncology, 2006. **33**(3): p. S9-S14.
457. Justus, C.R., et al., *In vitro Cell Migration and Invasion Assays.* Jove-Journal of Visualized Experiments, 2014(88).
458. Kramer, N., et al., *In vitro cell migration and invasion assays.* Mutation Research-Reviews in Mutation Research, 2013. **752**(1): p. 10-24.
459. Boyden, S., *Chemotactic Effect of Mixtures of Antibody and Antigen on Polymorphonuclear Leucocytes.* Journal of Experimental Medicine, 1962. **115**(3): p. 453-&.
460. Liang, C.C., A.Y. Park, and J.L. Guan, *In vitro scratch assay: a convenient and inexpensive method for analysis of cell migration in vitro.* Nature Protocols, 2007. **2**(2): p. 329-333.
461. Poujade, M., et al., *Collective migration of an epithelial monolayer in response to a model wound.* Proceedings of the National Academy of Sciences of the United States of America, 2007. **104**(41): p. 15988-15993.
462. Geback, T., et al., *TScratch: a novel and simple software tool for automated analysis of monolayer wound healing assays.* Biotechniques, 2009. **46**(4): p. 265-+.
463. Fronza, M., et al., *The scratch assay: A suitable in vitro tool for studying wound healing effects.* Planta Medica, 2008. **74**(9): p. 1125-1126.
464. Lo, C.M., C.R. Keese, and I. Giaever, *Impedance analysis of MDCK cells measured by electric cell-substrate impedance sensing.* Biophysical Journal, 1995. **69**(6): p. 2800-2807.

465. Fischer, E.G., A. Stingl, and C.J. Kirkpatrick, *Migration Assay for Endothelial-Cells in Multiwells - Application to Studies on the Effect of Opioids.* Journal of Immunological Methods, 1990. **128**(2): p. 235-239.
466. Zigmond, S.H., *Orientation Chamber in Chemotaxis.* Methods in Enzymology, 1988. **162**: p. 65-72.
467. Zicha, D., G.A. Dunn, and A.F. Brown, *A New Direct-Viewing Chemotaxis Chamber.* Journal of Cell Science, 1991. **99**: p. 769-775.
468. Chaubey, S., A.J. Ridley, and C.M. Wells, *Using the Dunn Chemotaxis Chamber to Analyze Primary Cell Migration in Real Time.* Cell Migration: Developmental Methods and Protocols, Second Edition, 2011. **769**: p. 41-51.
469. Nogalski, M.T., et al., *A Quantitative Evaluation of Cell Migration by the Phagokinetic Track Motility Assay.* Jove-Journal of Visualized Experiments, 2012(70).
470. Jaqaman, K., et al., *Robust single-particle tracking in live-cell time-lapse sequences.* Nature Methods, 2008. **5**(8): p. 695-702.
471. Zantl, R. and E. Horn, *Chemotaxis of Slow Migrating Mammalian Cells Analysed by Video Microscopy.* Cell Migration: Developmental Methods and Protocols, Second Edition, 2011. **769**: p. 191-203.
472. Orellana, R., et al., *Platelets enhance tissue factor protein and metastasis initiating cell markers, and act as chemoattractants increasing the migration of ovarian cancer cells.* Bmc Cancer, 2015. **15**.
473. Pignatelli, J., et al., *Invasive breast carcinoma cells from patients exhibit Mena(INV)- and macrophage-dependent transendothelial migration.* Science Signaling, 2014. **7**(353).
474. Kao, W.T., et al., *Investigation of MMP-2 and -9 in a highly invasive A431 tumor cell sub-line selected from a Boyden chamber assay.* Anticancer Research, 2008. **28**(4B): p. 2109-2120.
475. Gan, Y., et al., *Knockdown of HMGN5 suppresses the viability and invasion of human urothelial bladder cancer 5637 cells in vitro and in vivo (vol 32, 136, 2015).* Medical Oncology, 2015. **32**(5).
476. Yang, N., et al., *Silencing SOX2 Expression by RNA Interference Inhibits Proliferation, Invasion and Metastasis, and Induces Apoptosis through MAP4K4/JNK Signaling Pathway in Human Laryngeal Cancer TU212 Cells.* Journal of Histochemistry & Cytochemistry, 2015. **63**(9): p. 721-733.
477. Carter, J.C. and F.C. Church, *Mature breast adipocytes promote breast cancer cell motility.* Experimental and Molecular Pathology, 2012. **92**(3): p. 312-317.
478. Marshall, J., *Transwell (R) Invasion Assays.* Cell Migration: Developmental Methods and Protocols, Second Edition, 2011. **769**: p. 97-110.
479. Albini, A. and R. Benelli, *The chemoinvasion assay: a method to assess tumor and endothelial cell invasion and its modulation.* Nature Protocols, 2007. **2**(3): p. 504-511.
480. Ayala, I., et al., *Invadopodia: A guided tour.* European Journal of Cell Biology, 2006. **85**(3-4): p. 159-164.
481. Rocha, B., W.S. Haston, and A.A. Freitas, *Lymphocyte Migration into Collagen Gels - Role of Lymph.* Scandinavian Journal of Immunology, 1984. **19**(4): p. 297-305.
482. Nurmenniemi, S., et al., *A Novel Organotypic Model Mimics the Tumor Microenvironment.* American Journal of Pathology, 2009. **175**(3): p. 1281-1291.
483. Zaman, M.H., et al., *Migration of tumor cells in 3D matrices is governed by matrix stiffness along with cell-matrix adhesion and proteolysis (vol 103, pg 10889, 2006).* Proceedings of the National Academy of Sciences of the United States of America, 2006. **103**(37): p. 13897-13897.

484. Sutherland, R.M., J.A. Mccredie, and W.R. Inch, *Growth of Multicell Spheroids in Tissue Culture as a Model of Nodular Carcinomas.* Jnci-Journal of the National Cancer Institute, 1971. **46**(1): p. 113-+.
485. Naber, H.P.H., et al., *Spheroid Assay to Measure TGF-beta-induced Invasion.* Jove-Journal of Visualized Experiments, 2011(57).
486. Kunz-Schughart, L.A., et al., *A heterologous 3-D coculture model of breast tumor cells and fibroblasts to study tumor-associated fibroblast differentiation.* Experimental Cell Research, 2001. **266**(1): p. 74-86.
487. Hattermann, K., J. Held-Feindt, and R. Mentlein, *Spheroid confrontation assay: A simple method to monitor the three-dimensional migration of different cell types in vitro.* Annals of Anatomy-Anatomischer Anzeiger, 2011. **193**(3): p. 181-184.
488. Hirschhaeuser, F., et al., *Multicellular tumor spheroids: An underestimated tool is catching up again.* Journal of Biotechnology, 2010. **148**(1): p. 3-15.
489. Mikhail, A.S., S. Eetezadi, and C. Allen, *Multicellular Tumor Spheroids for Evaluation of Cytotoxicity and Tumor Growth Inhibitory Effects of Nanomedicines In Vitro: A Comparison of Docetaxel-Loaded Block Copolymer Micelles and Taxotere (R).* Plos One, 2013. **8**(4).
490. Ghosh, S., et al., *Use of multicellular tumor spheroids to dissect endothelial cell-tumor cell interactions: A role for T-cadherin in tumor angiogenesis.* Febs Letters, 2007. **581**(23): p. 4523-4528.
491. Decaestecker, C., et al., *Can anti-migratory drugs be screened in vitro? A review of 2D and 3D assays for the quantitative analysis of cell migration.* Medicinal Research Reviews, 2007. **27**(2): p. 149-176.
492. Kopetz, S., R. Lemos, and G. Powis, *The Promise of Patient-Derived Xenografts: The Best Laid Plans of Mice and Men.* Clinical Cancer Research, 2012. **18**(19): p. 5160-5162.
493. Julien, S., et al., *Characterization of a Large Panel of Patient-Derived Tumor Xenografts Representing the Clinical Heterogeneity of Human Colorectal Cancer.* Clinical Cancer Research, 2012. **18**(19): p. 5314-5328.
494. Suetsugu, A., et al., *Imageable Fluorescent Metastasis Resulting in Transgenic GFP Mice Orthotopically Implanted with Human-patient Primary Pancreatic Cancer Specimens.* Anticancer Research, 2012. **32**(4): p. 1175-1180.
495. Kim, M.P., et al., *Generation of orthotopic and heterotopic human pancreatic cancer xenografts in immunodeficient mice.* Nature Protocols, 2009. **4**(11): p. 1670-1680.
496. Kim, M.P., et al., *Molecular Profiling of Direct Xenograft Tumors Established from Human Pancreatic Adenocarcinoma After Neoadjuvant Therapy.* Annals of Surgical Oncology, 2012. **19**: p. S395-S403.
497. Troiani, T., et al., *Use of xenograft models for the selection of cancer treatments with the EGFR as an example.* Critical Reviews in Oncology Hematology, 2008. **65**(3): p. 200-211.
498. Fichtner, I., et al., *Anticancer drug response and expression of molecular marker in early-passage xenotransplanted colon carcinomas.* European Journal of Cancer, 2004. **40**(2): p. 298-307.
499. DeRose, Y.S., et al., *Tumor grafts derived from women with breast cancer authentically reflect tumor pathology, growth, metastasis and disease outcomes.* Nature Medicine, 2011. **17**(11): p. 1514-U227.
500. Monesi, V., *Differentiation of Argyrophil and Argentaffin Cells in Organotypic Cultures of Embryonic Chick Intestine.* Journal of Embryology and Experimental Morphology, 1960. **8**(3): p. 302-313.
501. Humpel, C., *Organotypic Brain Slice Cultures: A Review.* Neuroscience, 2015. **305**: p. 86-98.

502. Stoppini, L., P.A. Buchs, and D. Muller, *A Simple Method for Organotypic Cultures of Nervous-Tissue.* Journal of Neuroscience Methods, 1991. **37**(2): p. 173-182.
503. Jung, S., et al., *Brain tumor invasion model system using organotypic brain-slice culture as an alternative to in vivo model.* Journal of Cancer Research and Clinical Oncology, 2002. **128**(9): p. 469-476.
504. Morin-Brureau, M., et al., *Epileptiform Activity Induces Vascular Remodeling and Zonula Occludens 1 Downregulation in Organotypic Hippocampal Cultures: Role of VEGF Signaling Pathways.* Journal of Neuroscience, 2011. **31**(29): p. 10677-10688.
505. Pittet, M.J. and R. Weissleder, *Intravital Imaging.* Cell, 2011. **147**(5): p. 983-991.
506. Potter, S.M., et al., *Intravital imaging of green fluorescent protein using two-photon laser-scanning microscopy.* Gene, 1996. **173**(1): p. 25-31.
507. Schiessl, I.M. and H. Castrop, *Deep insights: intravital imaging with two-photon microscopy.* Pflugers Archiv-European Journal of Physiology, 2016. **468**(9): p. 1505-1516.
508. Medintz, I.L., et al., *Quantum dot bioconjugates for imaging, labelling and sensing.* Nature Materials, 2005. **4**(6): p. 435-446.
509. Cheung, K.J. and A.J. Ewald, *Illuminating breast cancer invasion: diverse roles for cell-cell interactions.* Current Opinion in Cell Biology, 2014. **30**: p. 99-111.
510. Condeelis, J. and J.E. Segall, *Intravital imaging of cell movement in tumours.* Nature Reviews Cancer, 2003. **3**(12): p. 921-930.
511. Hernandez, L., et al., *In Vivo Assay for Tumor Cell Invasion.* Chemotaxis: Methods and Protocols, 2009. **571**: p. 227-238.
512. Huang, C., K. Jacobson, and M.D. Schaller, *MAP kinases and cell migration.* Journal of Cell Science, 2004. **117**(20): p. 4619-4628.
513. Johnson, G.L. and R. Lapadat, *Mitogen-activated protein kinase pathways mediated by ERK, JNK, and p38 protein kinases.* Science, 2002. **298**(5600): p. 1911-1912.
514. Nolen, B., S. Taylor, and G. Ghosh, *Regulation of protein kinases: Controlling activity through activation segment conformation.* Molecular Cell, 2004. **15**(5): p. 661-675.
515. Gao, X., et al., *MAP4K4: an emerging therapeutic target in cancer.* Cell and Bioscience, 2016. **6**.
516. Delpire, E., *The mammalian family of sterile 20p-like protein kinases.* Pflugers Archiv-European Journal of Physiology, 2009. **458**(5): p. 953-967.
517. Dan, I., et al., *Molecular cloning of MINK, a novel member of mammalian GCK family kinases, which is up-regulated during postnatal mouse cerebral development.* Febs Letters, 2000. **469**(1): p. 19-23.
518. Wright, J.H., et al., *The STE20 kinase HGK is broadly expressed in human tumor cells and can modulate cellular transformation, invasion, and adhesion.* Molecular and Cellular Biology, 2003. **23**(6): p. 2068-2082.
519. Su, Y.C., et al., *NIK is a new Ste20-related kinase that binds NCK and MEKK1 and activates the SAPK/JNK cascade via a conserved regulatory domain.* Embo Journal, 1997. **16**(6): p. 1279-1290.
520. Kolch, W., *Coordinating ERK/MAPK signalling through scaffolds and inhibitors.* Nature Reviews Molecular Cell Biology, 2005. **6**(11): p. 827-837.
521. Liu, A.W., et al., *ShRNA-Targeted MAP4K4 Inhibits Hepatocellular Carcinoma Growth.* Clinical Cancer Research, 2011. **17**(4): p. 710-720.
522. Qiu, M.H., et al., *Expression and prognostic significance of MAP4K4 in lung adenocarcinoma.* Pathology Research and Practice, 2012. **208**(9): p. 541-548.
523. Rizzardi, A.E., et al., *Evaluation of protein biomarkers of prostate cancer aggressiveness.* Bmc Cancer, 2014. **14**.
524. Hao, J.M., et al., *A five-gene signature as a potential predictor of metastasis and survival in colorectal cancer.* Journal of Pathology, 2010. **220**(4): p. 475-489.

525. Liu, Y.F., et al., *Silencing of MAP4K4 by short hairpin RNA suppresses proliferation, induces G1 cell cycle arrest and induces apoptosis in gastric cancer cells.* Molecular Medicine Reports, 2016. **13**(1): p. 41-48.
526. Han, S.X., et al., *Lowered HGK expression inhibits cell invasion and adhesion in hepatocellular carcinoma cell line HepG2.* World Journal of Gastroenterology, 2010. **16**(36): p. 4541-4548.
527. Wang, B., et al., *MiR-194, commonly repressed in colorectal cancer, suppresses tumor growth by regulating the MAP4K4/c-Jun/MDM2 signaling pathway.* Cell Cycle, 2015. **14**(7): p. 1046-1058.
528. Haas, D.A., et al., *The Inflammatory Kinase MAP4K4 Promotes Reactivation of Kaposi's Sarcoma Herpesvirus and Enhances the Invasiveness of Infected Endothelial Cells.* Plos Pathogens, 2013. **9**(11).
529. Tang, X.Q., et al., *An RNA interference-based screen identifies MAP4K4/NIK as a negative regulator of PPAR gamma, adipogenesis, and insulin-responsive hexose transport.* Proceedings of the National Academy of Sciences of the United States of America, 2006. **103**(7): p. 2087-2092.
530. Hansen, C.G., T. Moroishi, and K.L. Guan, *YAP and TAZ: a nexus for Hippo signaling and beyond.* Trends in Cell Biology, 2015. **25**(9): p. 499-513.
531. Meng, Z.P., et al., *MAP4K family kinases act in parallel to MST1/2 to activate LATS1/2 in the Hippo pathway.* Nature Communications, 2015. **6**.
532. Danai, L.V., et al., *Map4k4 suppresses Srebp-1 and adipocyte lipogenesis independent of JNK signaling.* Journal of Lipid Research, 2013. **54**(10): p. 2697-2707.
533. Tesz, G.J., et al., *Tumor necrosis factor alpha (TNF alpha) stimulates Map4k4 expression through TNF alpha receptor 1 signaling to c-Jun and activating transcription factor 2.* Journal of Biological Chemistry, 2007. **282**(27): p. 19302-19312.
534. Baumgartner, M., et al., *The Nck-interacting kinase phosphorylates ERM proteins for formation of lamellipodium by growth factors.* Proc Natl Acad Sci U S A, 2006. **103**(36): p. 13391-6.
535. Xue, Y., et al., *Mesodermal patterning defect in mice lacking the Ste20 NCK interacting kinase (NIK).* Development, 2001. **128**(9): p. 1559-72.
536. Collins, C.S., et al., *A small interfering RNA screen for modulators of tumor cell motility identifies MAP4K4 as a promigratory kinase.* Proceedings of the National Academy of Sciences of the United States of America, 2006. **103**(10): p. 3775-3780.
537. Ndubaku, C.O., et al., *Structure-Based Design of GNE-495, a Potent and Selective MAP4K4 Inhibitor with Efficacy in Retinal Angiogenesis.* Acs Medicinal Chemistry Letters, 2015. **6**(8): p. 913-918.
538. Nakano, R., et al., *Fibroblast Growth Factor Receptor-2 Contributes to the Basic Fibroblast Growth Factor-Induced Neuronal Differentiation in Canine Bone Marrow Stromal Cells via Phosphoinositide 3-Kinase/Akt Signaling Pathway.* PLoS One, 2015. **10**(11): p. e0141581.
539. Beenken, A. and M. Mohammadi, *The FGF family: biology, pathophysiology and therapy.* Nat Rev Drug Discov, 2009. **8**(3): p. 235-53.
540. Ornitz, D.M. and N. Itoh, *Fibroblast growth factors.* Genome Biol, 2001. **2**(3): p. REVIEWS3005.
541. Potthoff, M.J., S.A. Kliewer, and D.J. Mangelsdorf, *Endocrine fibroblast growth factors 15/19 and 21: from feast to famine.* Genes Dev, 2012. **26**(4): p. 312-24.
542. Prudovsky, I., et al., *Protein-phospholipid interactions in nonclassical protein secretion: problem and methods of study.* Int J Mol Sci, 2013. **14**(2): p. 3734-72.
543. Prudovsky, I., et al., *The non-classical export routes: FGF1 and IL-1alpha point the way.* J Cell Sci, 2003. **116**(Pt 24): p. 4871-81.

544. Landriscina, M., et al., *S100A13 participates in the release of fibroblast growth factor 1 in response to heat shock in vitro.* J Biol Chem, 2001. **276**(25): p. 22544-52.
545. Wesche, J., et al., *FGF-1 and FGF-2 require the cytosolic chaperone Hsp90 for translocation into the cytosol and the cell nucleus.* J Biol Chem, 2006. **281**(16): p. 11405-12.
546. Krejci, P., et al., *Fibroblast growth factors 1, 2, 17, and 19 are the predominant FGF ligands expressed in human fetal growth plate cartilage.* Pediatr Res, 2007. **61**(3): p. 267-72.
547. Prudovsky, I., et al., *The intracellular translocation of the components of the fibroblast growth factor 1 release complex precedes their assembly prior to export.* J Cell Biol, 2002. **158**(2): p. 201-8.
548. Rodriguez-Enfedaque, A., et al., *FGF1 nuclear translocation is required for both its neurotrophic activity and its p53-dependent apoptosis protection.* Biochimica Et Biophysica Acta-Molecular Cell Research, 2009. **1793**(11): p. 1719-1727.
549. Bikfalvi, A., et al., *Biological roles of fibroblast growth factor-2.* Endocrine Reviews, 1997. **18**(1): p. 26-45.
550. Abraham, J.A., et al., *Human Basic Fibroblast Growth-Factor - Nucleotide-Sequence and Genomic Organization.* Embo Journal, 1986. **5**(10): p. 2523-2528.
551. Reiland, J. and A.C. Rapraeger, *Heparan-Sulfate Proteoglycan and Fgf Receptor Target Basic Fgf to Different Intracellular Destinations.* Journal of Cell Science, 1993. **105**: p. 1085-1093.
552. Itoh, N. and D.M. Ornitz, *Evolution of the Fgf and Fgfr gene families.* Trends in Genetics, 2004. **20**(11): p. 563-569.
553. Zhang, X.Q., et al., *Receptor specificity of the fibroblast growth factor family - The complete mammalian FGF family.* Journal of Biological Chemistry, 2006. **281**(23): p. 15694-15700.
554. Revest, J.M., L. DeMoerlooze, and C. Dickson, *Fibroblast growth factor 9 secretion is mediated by a non-cleaved amino-terminal signal sequence.* Journal of Biological Chemistry, 2000. **275**(11): p. 8083-8090.
555. Goetz, R., et al., *Molecular insights into the klotho-dependent, endocrine mode of action of fibroblast growth factor 19 subfamily members.* Molecular and Cellular Biology, 2007. **27**(9): p. 3417-3428.
556. Ding, X.S., et al., *beta Klotho Is Required for Fibroblast Growth Factor 21 Effects on Growth and Metabolism.* Cell Metabolism, 2012. **16**(3): p. 387-393.
557. Ho, H.K., et al., *Fibroblast growth factor receptor 4 regulates proliferation, anti-apoptosis and alpha-fetoprotein secretion during hepatocellular carcinoma progression and represents a potential target for therapeutic intervention.* Journal of Hepatology, 2009. **50**(1): p. 118-127.
558. Smallwood, P.M., et al., *Fibroblast growth factor (FGF) homologous factors: New members of the FGF family implicated in nervous system development.* Proceedings of the National Academy of Sciences of the United States of America, 1996. **93**(18): p. 9850-9857.
559. Givol, D. and A. Yayon, *Complexity of Fgf Receptors - Genetic-Basis for Structural Diversity and Functional Specificity.* Faseb Journal, 1992. **6**(15): p. 3362-3369.
560. Johnson, D.E. and L.T. Williams, *Structural and Functional Diversity in the Fgf Receptor Multigene Family.* Advances in Cancer Research, 1993. **60**: p. 1-41.
561. Olsen, S.K., et al., *Insights into the molecular basis for fibroblast growth factor receptor autoinhibition and ligand-binding promiscuity.* Proceedings of the National Academy of Sciences of the United States of America, 2004. **101**(4): p. 935-940.
562. Coutu, D.L. and J. Galipeau, *Roles of FGF signaling in stem cell self-renewal, senescence and aging.* Aging-Us, 2011. **3**(10): p. 920-933.

563. Yeh, B.K., et al., *Structural basis by which alternative splicing confers specificity in fibroblast growth factor receptors.* Proceedings of the National Academy of Sciences of the United States of America, 2003. **100**(5): p. 2266-2271.
564. Partanen, J., et al., *Fgfr-4, a Novel Acidic Fibroblast Growth-Factor Receptor with a Distinct Expression Pattern.* Embo Journal, 1991. **10**(6): p. 1347-1354.
565. Ornitz, D.M. and N. Itoh, *The Fibroblast Growth Factor signaling pathway.* Wiley Interdisciplinary Reviews-Developmental Biology, 2015. **4**(3): p. 215-266.
566. Ornitz, D.M., et al., *Receptor specificity of the fibroblast growth factor family.* Journal of Biological Chemistry, 1996. **271**(25): p. 15292-15297.
567. Ornitz, D.M., *FGFs, heparan sulfate and FGFRs: complex interactions essential for development.* Bioessays, 2000. **22**(2): p. 108-112.
568. Matsuo, I. and C. Kimura-Yoshida, *Extracellular modulation of Fibroblast Growth Factor signaling through heparan sulfate proteoglycans in mammalian development.* Current Opinion in Genetics & Development, 2013. **23**(4): p. 399-407.
569. Belov, A.A. and M. Mohammadi, *Molecular Mechanisms of Fibroblast Growth Factor Signaling in Physiology and Pathology.* Cold Spring Harbor Perspectives in Biology, 2013. **5**(6).
570. Patel, V.N., et al., *Specific heparan sulfate structures modulate FGF10-mediated submandibular gland epithelial morphogenesis and differentiation.* Journal of Biological Chemistry, 2008. **283**(14): p. 9308-9317.
571. Kuro-o, M., *Endocrine FGFs and Klothos: emerging concepts.* Trends in Endocrinology and Metabolism, 2008. **19**(7): p. 239-245.
572. Wu, D.Q., et al., *Characterization and Molecular-Cloning of a Putative Binding-Protein for Heparin-Binding Growth-Factors.* Journal of Biological Chemistry, 1991. **266**(25): p. 16778-16785.
573. Czubayko, F., et al., *A secreted FGF-binding protein can serve as the angiogenic switch in human cancer.* Nature Medicine, 1997. **3**(10): p. 1137-1140.
574. Dikic, I. and S. Giordano, *Negative receptor signalling.* Current Opinion in Cell Biology, 2003. **15**(2): p. 128-135.
575. Guy, G.R., et al., *Sprouty proteins: modified modulators, matchmakers or missing links?* Journal of Endocrinology, 2009. **203**(2): p. 191-202.
576. Yang, X.H., et al., *Spry1 and Spry4 Differentially Regulate Human Aortic Smooth Muscle Cell Phenotype via Akt/FoxO/Myocardin Signaling.* Plos One, 2013. **8**(3).
577. Tsang, M., et al., *Identification of Sef, a novel modulator of FGF signalling.* Nature Cell Biology, 2002. **4**(2): p. 165-169.
578. Torii, S., et al., *Sef is a spatial regulator for Ras/MAP kinase signaling.* Developmental Cell, 2004. **7**(1): p. 33-44.
579. Kovalenko, D., et al., *A role for extracellular and transmembrane domains of Sef in Sef-mediated inhibition of FGF signaling.* Cellular Signalling, 2006. **18**(11): p. 1958-1966.
580. Li, C.Y., et al., *Dusp6 (Mkp3) is a negative feedback regulator of FGF-stimulated ERK signaling during mouse development.* Development, 2007. **134**(1): p. 167-176.
581. Dufour, C., et al., *FGFR2-Cbl interaction in lipid rafts triggers attenuation of PI3K/Akt signaling and osteoblast survival.* Bone, 2008. **42**(6): p. 1032-9.
582. Trueb, B., *Biology of FGFRL1, the fifth fibroblast growth factor receptor.* Cellular and Molecular Life Sciences, 2011. **68**(6): p. 951-964.
583. Steinberg, F., et al., *The FGFRL1 Receptor Is Shed from Cell Membranes, Binds Fibroblast Growth Factors (FGFs), and Antagonizes FGF Signaling in Xenopus Embryos.* Journal of Biological Chemistry, 2010. **285**(3): p. 2193-2202.

584. Zhou, W.C., et al., *FGF-receptor substrate 2 functions as a molecular sensor integrating external regulatory signals into the FGF pathway.* Cell Research, 2009. **19**(10): p. 1165-1177.
585. Lax, I., et al., *The docking protein FRS2alpha controls a MAP kinase-mediated negative feedback mechanism for signaling by FGF receptors.* Mol Cell, 2002. **10**(4): p. 709-19.
586. Furdui, C.M., et al., *Autophosphorylation of FGFR1 kinase is mediated by a sequential and precisely ordered reaction.* Molecular Cell, 2006. **21**(5): p. 711-717.
587. Eswarakumar, V.P., I. Lax, and J. Schlessinger, *Cellular signaling by fibroblast growth factor receptors.* Cytokine & Growth Factor Reviews, 2005. **16**(2): p. 139-149.
588. Larsson, H., et al., *Fibroblast growth factor receptor-1-mediated endothelial cell proliferation is dependent on the Src homology (SH) 2/SH3 domain-containing adaptor protein Crk.* Journal of Biological Chemistry, 1999. **274**(36): p. 25726-25734.
589. Kouhara, H., et al., *A lipid-anchored Grb2-binding protein that links FGF-receptor activation to the Ras/MAPK signaling pathway.* Cell, 1997. **89**(5): p. 693-702.
590. Manning, B.D. and L.C. Cantley, *AKT/PKB signaling: Navigating downstream.* Cell, 2007. **129**(7): p. 1261-1274.
591. Lamothe, B., et al., *The docking protein Gab1 is an essential component of an indirect mechanism for fibroblast growth factor stimulation of the phosphatidylinositol 3-kinase/Akt antiapoptotic pathway.* Molecular and Cellular Biology, 2004. **24**(13): p. 5657-5666.
592. Mohammadi, M., et al., *A Tyrosine-Phosphorylated Carboxy-Terminal Peptide of the Fibroblast Growth-Factor Receptor (Flg) Is a Binding-Site for the Sh2 Domain of Phospholipase C-Gamma-1.* Molecular and Cellular Biology, 1991. **11**(10): p. 5068-5078.
593. Spivakkroizman, T., et al., *Point Mutation in the Fibroblast Growth-Factor Receptor Eliminates Phosphatidylinositol Hydrolysis without Affecting Neuronal Differentiation of Pc12 Cells.* Journal of Biological Chemistry, 1994. **269**(20): p. 14419-14423.
594. Kolkova, K., et al., *Neural cell adhesion molecule-stimulated neurite outgrowth depends on activation of protein kinase C and the Ras-mitogen-activated protein kinase pathway.* Journal of Neuroscience, 2000. **20**(6): p. 2238-2246.
595. Dudka, A.A., S.M.M. Sweet, and J.K. Heath, *Signal Transducers and Activators of Transcription-3 Binding to the Fibroblast Growth Factor Receptor Is Activated by Receptor Amplification.* Cancer Research, 2010. **70**(8): p. 3391-3401.
596. Sahni, M., et al., *FGF signaling inhibits chondrocyte proliferation and regulates bone development through the STAT-1 pathway.* Genes & Development, 1999. **13**(11): p. 1361-1366.
597. Yang, X.H., et al., *Signal Transducers and Activators of Transcription Mediate Fibroblast Growth Factor-Induced Vascular Endothelial Morphogenesis.* Cancer Research, 2009. **69**(4): p. 1668-1677.
598. Mason, I., *Initiation to end point: the multiple roles of fibroblast growth factors in neural development.* Nature Reviews Neuroscience, 2007. **8**(8): p. 583-596.
599. Gotoh, N., *Regulation of growth factor signaling by FRS2 family docking/scaffold adaptor proteins.* Cancer Science, 2008. **99**(7): p. 1319-1325.
600. Csiszar, A., *Structural and functional diversity of adaptor proteins involved in tyrosine kinase signalling.* Bioessays, 2006. **28**(5): p. 465-479.
601. Sato, T. and N. Gotoh, *The FRS2 family of docking/scaffolding adaptor proteins as therapeutic targets of cancer treatment.* Expert Opinion on Therapeutic Targets, 2009. **13**(6): p. 689-700.

602. Gotoh, N., et al., *FRS2 family docking proteins with overlapping roles in activation of MAP kinase have distinct spatial-temporal patterns of expression of their transcripts.* Febs Letters, 2004. **564**(1-2): p. 14-18.
603. Cotton, L.M., M.K. O'Bryan, and B.T. Hinton, *Cellular signaling by fibroblast growth factors (FGFs) and their receptors (FGFRs) in male reproduction.* Endocrine Reviews, 2008. **29**(2): p. 193-216.
604. Xu, H., K.W. Lee, and M. Goldfarb, *Novel recognition motif on fibroblast growth factor receptor mediates direct association and activation of SNT adapter proteins.* Journal of Biological Chemistry, 1998. **273**(29): p. 17987-17990.
605. Ong, S.H., et al., *FRS2 proteins recruit intracellular signaling pathways by binding to diverse targets on fibroblast growth factor and nerve growth factor receptors.* Molecular and Cellular Biology, 2000. **20**(3): p. 979-989.
606. Burgar, H.R., et al., *Association of the signaling adaptor FRS2 with fibroblast growth factor receptor 1 (Fgfr1) is mediated by alternative splicing of the juxtamembrane domain.* Journal of Biological Chemistry, 2002. **277**(6): p. 4018-4023.
607. Zeng, L., et al., *Structural insights into FRS2 alpha PTB domain recognition by neurotrophin receptor TrkB.* Proteins-Structure Function and Bioinformatics, 2014. **82**(7): p. 1534-1541.
608. Yan, K.S., et al., *FRS2 PTB domain conformation regulates interactions with divergent neurotrophic receptors.* Journal of Biological Chemistry, 2002. **277**(19): p. 17088-17094.
609. Li, X., et al., *FRS2-dependent SRC activation is required for fibroblast growth factor receptor-induced phosphorylation of sprouty and suppression of ERK activity.* Journal of Cell Science, 2004. **117**(25): p. 6007-6017.
610. Yan, K.S., et al., *FRS2 PTB domain conformation regulates interactions with divergent neurotrophic receptors.* J Biol Chem, 2002. **277**(19): p. 17088-94.
611. Kameda, Y., et al., *FRS2 alpha(2F/2F) mice lack carotid body and exhibit abnormalities of the superior cervical sympathetic ganglion and carotid sinus nerve.* Developmental Biology, 2008. **314**(1): p. 236-247.
612. Yamamoto, S., et al., *Essential role of Shp2-binding sites on FRS2 alpha for corticogenesis and for FGF2-dependent proliferation of neural progenitor cells.* Proceedings of the National Academy of Sciences of the United States of America, 2005. **102**(44): p. 15983-15988.
613. Zhang, Y.Y., et al., *Role of epithelial cell fibroblast growth factor receptor substrate 2 alpha in prostate development, regeneration and tumorigenesis.* Development, 2008. **135**(4): p. 775-784.
614. Valencia, T., et al., *Role and expression of FRS2 and FRS3 in prostate cancer.* Bmc Cancer, 2011. **11**.
615. Luo, L.Y., et al., *The Tyrosine Kinase Adaptor Protein FRS2 Is Oncogenic and Amplified in High-Grade Serous Ovarian Cancer.* Molecular Cancer Research, 2015. **13**(3): p. 502-509.
616. Retting, K.N., et al., *BMP canonical Smad signaling through Smad1 and Smad5 is required for endochondral bone formation.* Development, 2009. **136**(7): p. 1093-1104.
617. Khokha, M.K., et al., *Gremlin is the BMP antagonist required for maintenance of Shh and Fgf signals during limb patterning.* Nature Genetics, 2003. **34**(3): p. 303-307.
618. Jin, M., X.L. Du, and L. Chen, *Cross-talk between FGF and other cytokine signalling pathways during endochondral bone development.* Cell Biology International, 2012. **36**(8): p. 691-696.

619. Katoh, M. and M. Katoh, *Cross-talk of WNT and FGF signaling pathways at GSK3beta to regulate beta-catenin and SNAIL signaling cascades.* Cancer Biol Ther, 2006. **5**(9): p. 1059-64.
620. Zhou, Y., et al., *FGF receptor-dependent, integrin-independent phosphorylation of p38, JNK and ERK MAPKs following mechanical stimulation of human articular chondrocytes in primary monolayer cell culture.* International Journal of Experimental Pathology, 2010. **91**(2): p. A28-A28.
621. Hutley, L., et al., *Fibroblast growth factor 1 - A key regulator of human adipogenesis.* Diabetes, 2004. **53**(12): p. 3097-3106.
622. Holland, E.C. and H.E. Varmus, *Basic fibroblast growth factor induces cell migration and proliferation after glia-specific gene transfer in mice.* Proceedings of the National Academy of Sciences of the United States of America, 1998. **95**(3): p. 1218-1223.
623. Tanaka, S., et al., *Promotion of trophoblast stem cell proliferation by FGF4.* Science, 1998. **282**(5396): p. 2072-5.
624. Finch, P.W., et al., *Human Kgf Is Fgf-Related with Properties of a Paracrine Effector of Epithelial-Cell Growth.* Science, 1989. **245**(4919): p. 752-755.
625. Thomson, A.A. and G.R. Cunha, *Prostatic growth and development are regulated by FGF10.* Development, 1999. **126**(16): p. 3693-3701.
626. Ohbayashi, N., et al., *FGF18 is required for normal cell proliferation and differentiation during osteogenesis and chondrogenesis.* Genes & Development, 2002. **16**(7): p. 870-879.
627. Hossain, W.A. and D.K. Morest, *Fibroblast growth factors (FGF-1, FGF-2) promote migration and neurite growth of mouse cochlear ganglion cells in vitro: Immunohistochemistry and antibody perturbation.* Journal of Neuroscience Research, 2000. **62**(1): p. 40-55.
628. Webb, S.E., et al., *Fibroblast growth factors 2 and 4 stimulate migration of mouse embryonic limb myogenic cells.* Developmental Dynamics, 1997. **209**(2): p. 206-216.
629. Tsuboi, R., et al., *Keratinocyte Growth-Factor (Fgf-7) Stimulates Migration and Plasminogen-Activator Activity of Normal Human Keratinocytes.* Journal of Investigative Dermatology, 1993. **101**(1): p. 49-53.
630. Kubota, Y. and K. Ito, *Chemotactic migration of mesencephalic neural crest cells in the mouse.* Developmental Dynamics, 2000. **217**(2): p. 170-179.
631. Murphy, M., J. Drago, and P.F. Bartlett, *Fibroblast Growth-Factor Stimulates the Proliferation and Differentiation of Neural Precursor Cells-Invitro.* Journal of Neuroscience Research, 1990. **25**(4): p. 463-475.
632. Werner, S., et al., *Targeted Expression of a Dominant-Negative Fgf Receptor Mutant in the Epidermis of Transgenic Mice Reveals a Role of Fgf in Keratinocyte Organization and Differentiation.* Embo Journal, 1993. **12**(7): p. 2635-2643.
633. Takagi, Y., et al., *Dopaminergic neurons generated from monkey embryonic stem cells function in a Parkinson primate model.* Journal of Clinical Investigation, 2005. **115**(1): p. 102-109.
634. Javerzat, S., P. Auguste, and A. Bikfalvi, *The role of fibroblast growth factors in vascular development.* Trends in Molecular Medicine, 2002. **8**(10): p. 483-489.
635. Reuss, B. and O. von Bohlen und Halbach, *Fibroblast growth factors and their receptors in the central nervous system.* Cell and Tissue Research, 2003. **313**(2): p. 139-157.
636. Arnaud, E., et al., *A new 34-kilodalton isoform of human fibroblast growth factor 2 is cap dependently synthesized by using a non-AUG start codon and behaves as a survival factor.* Molecular and Cellular Biology, 1999. **19**(1): p. 505-514.

637. Stock, A., et al., *Localization of Acidic Fibroblast Growth-Factor in Specific Subcortical Neuronal Populations.* Journal of Neuroscience, 1992. **12**(12): p. 4688-4700.
638. Eckenstein, F., W.R. Woodward, and R. Nishi, *Differential Localization and Possible Functions of Afgf and Bfgf in the Central and Peripheral Nervous Systems.* Fibroblast Growth Factor Family, 1991. **638**: p. 348-360.
639. Ernfors, P., et al., *Developmental and Regional Expression of Basic Fibroblast Growth-Factor Messengerrna in the Rat Central-Nervous-System.* Journal of Neuroscience Research, 1990. **27**(1): p. 10-15.
640. Gomezpinilla, F., J.W.K. Lee, and C.W. Cotman, *Distribution of Basic Fibroblast Growth-Factor in the Developing Rat-Brain.* Neuroscience, 1994. **61**(4): p. 911-923.
641. Belluardo, N., et al., *Comparative localization of fibroblast growth factor receptor-1, -2, and -3 mRNAs in the rat brain: In situ hybridization analysis.* Journal of Comparative Neurology, 1997. **379**(2): p. 226-246.
642. Fuhrmann, V., et al., *Fibroblast growth factor receptor 4 (FGFR4) is expressed in adult rat and human retinal photoreceptors and neurons.* Journal of Molecular Neuroscience, 1999. **13**(1-2): p. 187-197.
643. Akl, M.R., et al., *Molecular and clinical significance of fibroblast growth factor 2 (FGF2 /bFGF) in malignancies of solid and hematological cancers for personalized therapies.* Oncotarget, 2016. **7**(28): p. 44735-44762.
644. Ford-Perriss, M., H. Abud, and M. Murphy, *Fibroblast growth factors in the developing central nervous system.* Clinical and Experimental Pharmacology and Physiology, 2001. **28**(7): p. 493-503.
645. Bouvier, M.M. and C. Mytilineou, *Basic Fibroblast Growth-Factor Increases Division and Delays Differentiation of Dopamine Precursors in-Vitro.* Journal of Neuroscience, 1995. **15**(11): p. 7141-7149.
646. Deloulme, J.C., et al., *Effects of Basic Fibroblast Growth-Factor on the Development of Gabaergic Neurons in Culture.* Neuroscience, 1991. **42**(2): p. 561-568.
647. Arsenijevic, Y., et al., *Insulin-like growth factor-I is necessary for neural stem cell proliferation and demonstrates distinct actions of epidermal growth factor and fibroblast growth factor-2.* J Neurosci, 2001. **21**(18): p. 7194-202.
648. Nakagami, Y., H. Saito, and N. Matsuki, *Basic fibroblast growth factor and brain-derived neurotrophic factor promote survival and neuronal circuit formation in organotypic hippocampal culture.* Japanese Journal of Pharmacology, 1997. **75**(4): p. 319-326.
649. Kalil, K., F. Tang, and E.W. Dent, *Common mechanisms underlying growth cone guidance and axon branching.* Developmental Biology, 2002. **247**(2): p. 482-483.
650. Patel, M.N. and J.O. Mcnamara, *Selective Enhancement of Axonal Branching of Cultured Dentate Gyrus Neurons by Neurotrophic Factors.* Neuroscience, 1995. **69**(3): p. 763-770.
651. Aoyagi, A., et al., *Characterization of Basic Fibroblast Growth Factor-Mediated Acceleration of Axonal Branching in Cultured Rat Hippocampal-Neurons.* Brain Research, 1994. **661**(1-2): p. 117-126.
652. Mattson, M.P., et al., *Fibroblast Growth-Factor and Glutamate - Opposing Roles in the Generation and Degeneration of Hippocampal Neuroarchitecture.* Journal of Neuroscience, 1989. **9**(11): p. 3728-3740.
653. Perkins, L.A. and L.D. Cain, *Basic Fibroblast Growth-Factor (Bfgf) Increases the Survival of Embryonic and Postnatal Basal Forebrain Cholinergic Neurons in Primary Culture.* International Journal of Developmental Neuroscience, 1995. **13**(1): p. 51-61.
654. Frautschy, S.A., P.A. Walicke, and A. Baird, *Localization of Basic Fibroblast Growth-Factor and Its Messenger-Rna after Cns Injury.* Brain Research, 1991. **553**(2): p. 291-299.

655. Ogata, N., et al., *Expression of basic fibroblast growth factor mRNA in developing choroidal neovascularization.* Current Eye Research, 1996. **15**(10): p. 1008-1018.
656. Cuevas, P., et al., *Intravenous fibroblast growth factor penetrates the blood-brain barrier and protects hippocampal neurons against ischemia-reperfusion injury.* Surgical Neurology, 1998. **49**(1): p. 77-83.
657. Sadohara, T., et al., *Keratinocyte growth factor prevents ischemia-induced delayed neuronal death in the hippocampal CA I field of the gerbil brain.* Neuroreport, 2001. **12**(1): p. 71-76.
658. Martinez, G., et al., *Fibroblast growth factor-2 and transforming growth factor-beta-1 immunostaining in rat brain after cerebral postischemic reperfusion.* Journal of Neuroscience Research, 2001. **63**(2): p. 136-142.
659. Ou-Yang, W., et al., *Alteration of basic fibroblast growth factor expression in rat during cerebral ischemia.* Acta Pharmacologica Sinica, 2000. **21**(4): p. 296-300.
660. Ishiyama, J., H. Saito, and K. Abe, *Epidermal growth factor and basic fibroblast growth factor promote the generation of long-term potentiation in the dentate gyrus of anaesthetized rats.* Neurosci Res, 1991. **12**(3): p. 403-11.
661. Ford-Perriss, M., H. Abud, and M. Murphy, *Fibroblast growth factors in the developing central nervous system.* Clin Exp Pharmacol Physiol, 2001. **28**(7): p. 493-503.
662. Turner, N. and R. Grose, *Fibroblast growth factor signalling: from development to cancer.* Nature Reviews Cancer, 2010. **10**(2): p. 116-129.
663. Kunii, K., et al., *FGFR2-amplified gastric cancer cell lines require FGFR2 and Erbb3 signaling for growth and survival (vol 68, pg 2340, 2008).* Cancer Research, 2008. **68**(9): p. 3549-3549.
664. Jacquemier, J., et al., *Expression of the Fgfr1 Gene in Human Breast-Carcinoma Cells.* International Journal of Cancer, 1994. **59**(3): p. 373-378.
665. Freier, K., et al., *Recurrent FGFR 1 amplification and high FGFR1 protein expression in oral squamous cell carcinoma (OSCC).* Oral Oncology, 2007. **43**(1): p. 60-66.
666. Gorringe, K.L., et al., *High-resolution single nucleotide polymorphism array analysis of epithelial ovarian cancer reveals numerous microdeletions and amplifications.* Clinical Cancer Research, 2007. **13**(16): p. 4731-4739.
667. Cappellen, D., et al., *Frequent activating mutations of FGFR3 in human bladder and cervix carcinomas.* Nature Genetics, 1999. **23**(1): p. 18-20.
668. Rosty, C., et al., *Clinical and biological characteristics of cervical neoplasias with FGFR3 mutation.* Molecular Cancer, 2005. **4**.
669. Hernandez, S., et al., *FGFR3 mutations in prostate cancer: association with low-grade tumors.* Modern Pathology, 2009. **22**(6): p. 848-856.
670. Logie, A., et al., *Activating mutations of the tyrosine kinase receptor FGFR3 are associated with benign skin tumors in mice and humans.* Human Molecular Genetics, 2005. **14**(9): p. 1153-1160.
671. Chesi, M., et al., *Activated fibroblast growth factor receptor 3 is an oncogene that contributes to tumor progression in multiple myeloma.* Blood, 2001. **97**(3): p. 729-736.
672. Ornitz, D.M. and P.J. Marie, *FGF signaling pathways in endochondral and intramembranous bone development and human genetic disease.* Genes & Development, 2002. **16**(12): p. 1446-1465.
673. Bryant, D.M., F.G. Wylie, and J.L. Stow, *Regulation of endocytosis, nuclear translocation, and signaling of fibroblast growth factor receptor 1 by E-cadherin.* Molecular Biology of the Cell, 2005. **16**(1): p. 14-23.

674. Wang, Y.P. and D. Becker, *Antisense targeting of basic fibroblast growth factor and fibroblast growth factor receptor-1 in human melanomas blocks intratumoral angiogenesis and tumor growth.* Nature Medicine, 1997. **3**(8): p. 887-893.
675. Giri, D., F. Ropiquet, and M. Ittmann, *Alterations in expression of basic fibroblast growth factor (FGF) 2 and its receptor FGFR-1 in human prostate cancer.* Clinical Cancer Research, 1999. **5**(5): p. 1063-1071.
676. Nicholes, K., et al., *A mouse model of hepatocellular carcinoma - Ectopic expression of fibroblast growth factor 19 in skeletal muscle of transgenic mice.* American Journal of Pathology, 2002. **160**(6): p. 2295-2307.
677. Desnoyers, L., et al., *Targeting FGF19 inhibits tumor growth in colon cancer xenograft and FGF19 transgenic hepatocellular carcinoma models.* Oncogene, 2008. **27**(1): p. 85-97.
678. Pai, R., et al., *Inhibition of fibroblast growth factor 19 reduces tumor growth by modulating beta-catenin signaling.* Cancer Research, 2008. **68**(13): p. 5086-5095.
679. Easton, D.F., et al., *Genome-wide association study identifies novel breast cancer susceptibility loci.* Nature, 2007. **447**(7148): p. 1087-U7.
680. Bange, J., et al., *Cancer progression and tumor cell motility are associated with the FGFR4 Arg(388) allele.* Cancer Research, 2002. **62**(3): p. 840-847.
681. Spinola, M., et al., *FGFR4 Gly388Arg polymorphism and prognosis of breast and colorectal cancer.* Oncology Reports, 2005. **14**(2): p. 415-419.
682. Avet-Loiseau, H., et al., *High incidence of translocations t(11;14)(q13;q32) and t(4;14)(p16;q32) in patients with plasma cell malignancies.* Cancer Research, 1998. **58**(24): p. 5640-5645.
683. Chesi, M., et al., *Frequent translocation t(4;14)(p16.3;q32.3) in multiple myeloma is associated with increased expression and activating mutations of fibroblast growth factor receptor 3.* Nature Genetics, 1997. **16**(3): p. 260-264.
684. Xiao, S., et al., *FGFR1 is fused with a novel zinc-finger gene, ZNF198, in the t(8;13) leukaemia/lymphoma syndrome.* Nature Genetics, 1998. **18**(1): p. 84-87.
685. Roumiantsev, S., et al., *Distinct stem cell myeloproliferative/T lymphoma syndromes induced by ZNF198-FGFR1 and BCR-FGFR1 fusion genes from 8p11 translocations.* Cancer Cell, 2004. **5**(3): p. 287-298.
686. Pardo, O.E., et al., *Fibroblast growth factor-2 induces translational regulation of Bcl-X-L and Bcl-2 via a MEK-dependent pathway. CORRELATION WITH RESISTANCE TO ETOPOSIDE-INDUCED APOPTOSIS (vol 277, pg 12040, 2002).* Journal of Biological Chemistry, 2015. **290**(25): p. 15390-15390.
687. Memarzadeh, S., et al., *Enhanced paracrine FGF10 expression promotes formation of multifocal prostate adenocarcinoma and an increase in epithelial androgen receptor.* Cancer Cell, 2007. **12**(6): p. 572-585.
688. Ruotsalainen, T., et al., *High pretreatment serum concentration of basic fibroblast growth factor is a predictor of poor prognosis in small cell lung cancer.* Cancer Epidemiology Biomarkers & Prevention, 2002. **11**(11): p. 1492-1495.
689. Tomlinson, D.C., et al., *Fibroblast Growth Factor Receptor 1 Promotes Proliferation and Survival via Activation of the Mitogen-Activated Protein Kinase Pathway in Bladder Cancer.* Cancer Research, 2009. **69**(11): p. 4613-4620.
690. Welm, B.E., et al., *Inducible dimerization of FGFR1: development of a mouse model to analyze progressive transformation of the mammary gland.* Journal of Cell Biology, 2002. **157**(4): p. 703-714.
691. Acevedo, V.D., et al., *Inducible FGFR-1 activation leads to irreversible prostate adenocarcinoma and an epithelial-to-mesenchymal transition.* Cancer Cell, 2007. **12**(6): p. 559-571.

692. Aman, A. and T. Piotrowski, *Wnt/beta-Catenin and Fgf Signaling Control Collective Cell Migration by Restricting Chemokine Receptor Expression.* Developmental Cell, 2008. **15**(5): p. 749-761.
693. Presta, M., et al., *Fibroblast growth factor/fibroblast growth factor receptor system in angiogenesis.* Cytokine & Growth Factor Reviews, 2005. **16**(2): p. 159-178.
694. Presta, M., et al., *Basic Fibroblast Growth-Factor Requires a Long-Lasting Activation of Protein-Kinase-C to Induce Cell-Proliferation in Transformed Fetal Bovine Aortic Endothelial-Cells.* Cell Regulation, 1991. **2**(9): p. 719-726.
695. Fogarty, M.P., et al., *Fibroblast growth factor blocks Sonic hedgehog signaling in neuronal precursors and tumor cells.* Proceedings of the National Academy of Sciences of the United States of America, 2007. **104**(8): p. 2973-2978.
696. Ricol, D., et al., *Tumour suppressive properties of fibroblast growth factor receptor 2-IIIb in human bladder cancer.* Oncogene, 1999. **18**(51): p. 7234-7243.
697. Zhang, Y., et al., *Growth inhibition by keratinocyte growth factor receptor of human salivary adenocarcinoma cells through induction of differentiation and apoptosis.* Proceedings of the National Academy of Sciences of the United States of America, 2001. **98**(20): p. 11336-11340.
698. Gartside, M.G., et al., *Loss-of-Function Fibroblast Growth Factor Receptor-2 Mutations in Melanoma.* Molecular Cancer Research, 2009. **7**(1): p. 41-54.
699. Boismenu, R. and W.L. Havran, *Modulation of Epithelial-Cell Growth by Intraepithelial Gamma-Delta T-Cells.* Science, 1994. **266**(5188): p. 1253-1255.
700. Touat, M., et al., *Targeting FGFR Signaling in Cancer.* Clinical Cancer Research, 2015. **21**(12): p. 2684-2694.
701. Soria, J.C., et al., *A phase I/IIa study evaluating the safety, efficacy, pharmacokinetics, and pharmacodynamics of lucitanib in advanced solid tumors.* Journal of Clinical Oncology, 2014. **32**(15).
702. Andre, F., et al., *Targeting FGFR with Dovitinib (TKI258): Preclinical and Clinical Data in Breast Cancer.* Clinical Cancer Research, 2013. **19**(13): p. 3693-3702.
703. Dienstmann, R., et al., *Genomic aberrations in the FGFR pathway: opportunities for targeted therapies in solid tumors.* Annals of Oncology, 2014. **25**(3): p. 552-563.
704. Bahleda, R., et al., *Phase 1 study of JNJ-42756493, a pan-fibroblast growth factor receptor (FGFR) inhibitor, in patients with advanced solid tumors.* Journal of Clinical Oncology, 2014. **32**(15).
705. Di Stefano, A.L., et al., *Detection, Characterization, and Inhibition of FGFR-TACC Fusions in IDH Wild-type Glioma.* Clinical Cancer Research, 2015. **21**(14): p. 3307-3317.
706. Andre, F., et al., *Results of a phase I study of AZD4547, an inhibitor of fibroblast growth factor receptor (FGFR), in patients with advanced solid tumors.* Cancer Research, 2013. **73**(8).
707. Sequist, L.V., et al., *Phase I study of BGJ398, a selective pan-FGFR inhibitor in genetically preselected advanced solid tumors.* Cancer Research, 2014. **74**(19).
708. Wolf, J., et al., *A phase I dose escalation study of NVP-BGJ398, a selective pan FGFR inhibitor in genetically preselected advanced solid tumors.* Cancer Research, 2012. **72**.
709. Ochiiwa, H., et al., *TAS-120, a highly potent and selective irreversible FGFR inhibitor, is effective in tumors harboring various FGFR gene abnormalities.* Molecular Cancer Therapeutics, 2013. **12**(11).
710. Odonnell, P., et al., *A Phase I Dose-escalation Study of MFGR1877S, a Human Monoclonal Anti-fibroblast Growth Factor Receptor 3 (FGFR3) Antibody, in Patients (pts) with Advanced Solid Tumors.* European Journal of Cancer, 2012. **48**: p. 191-192.

711. Bellovin, D.I., et al., *FP-1039/GSK3052230, an FGF ligand trap, enhances VEGF antagonist therapy in preclinical models of RCC and HCC.* Cancer Research, 2014. **74**(19).
712. Weiss, A. and L. Attisano, *The TGFbeta Superfamily Signaling Pathway.* Wiley Interdisciplinary Reviews-Developmental Biology, 2013. **2**(1): p. 47-63.
713. Gordon, K.J. and G.C. Blobe, *Role of transforming growth factor-beta superfamily signaling pathways in human disease.* Biochimica Et Biophysica Acta-Molecular Basis of Disease, 2008. **1782**(4): p. 197-228.
714. Wrana, J.L., et al., *Mechanism of Activation of the Tgf-Beta Receptor.* Nature, 1994. **370**(6488): p. 341-347.
715. Kubiczkova, L., et al., *TGF-beta - an excellent servant but a bad master.* Journal of Translational Medicine, 2012. **10**.
716. Cheifetz, S., et al., *The Transforming Growth-Factor-Beta System, a Complex Pattern of Cross-Reactive Ligands and Receptors.* Cell, 1987. **48**(3): p. 409-415.
717. Derynck, R., et al., *Human Transforming Growth Factor-Beta Complementary-DNA Sequence and Expression in Normal and Transformed-Cells.* Nature, 1985. **316**(6030): p. 701-705.
718. Dickinson, M.E., et al., *Chromosomal localization of seven members of the murine TGF-beta superfamily suggests close linkage to several morphogenetic mutant loci.* Genomics, 1990. **6**(3): p. 505-20.
719. Demartin, R., et al., *Complementary-DNA for Human Glioblastoma-Derived T-Cell Suppressor Factor, a Novel Member of the Transforming Growth Facto-Beta Gene Family.* Embo Journal, 1987. **6**(12): p. 3673-3677.
720. Tendijke, P., et al., *Identification of Another Member of the Transforming Growth-Factor Type-Beta-Gene Family.* Proceedings of the National Academy of Sciences of the United States of America, 1988. **85**(13): p. 4715-4719.
721. Flanders, K.C., et al., *Localization and Actions of Transforming Growth Factor-Beta-S in the Embryonic Nervous-System.* Development, 1991. **113**(1): p. 183-&.
722. Proetzel, G., et al., *Transforming Growth Factor-Beta-3 Is Required for Secondary Palate Fusion.* Nature Genetics, 1995. **11**(4): p. 409-414.
723. Kaartinen, V., et al., *Abnormal Lung Development and Cleft-Palate in Mice Lacking Tgf-Beta-3 Indicates Defects of Epithelial-Mesenchymal Interaction.* Nature Genetics, 1995. **11**(4): p. 415-421.
724. Massague, J., *Receptors for the Tgf-Beta Family.* Cell, 1992. **69**(7): p. 1067-1070.
725. Lyons, R.M., J. Keskioja, and H.L. Moses, *Proteolytic Activation of Latent Transforming Growth Factor-Beta from Fibroblast-Conditioned Medium.* Journal of Cell Biology, 1988. **106**(5): p. 1659-1665.
726. Lu, S.L., et al., *Genomic structure of the transforming growth factor beta type II receptor gene and its mutations in hereditary nonpolyposis colorectal cancers.* Cancer Research, 1996. **56**(20): p. 4595-4598.
727. Cheifetz, S., J.L. Andres, and J. Massague, *The Transforming Growth Factor-Beta Receptor Type-Iii Is a Membrane Proteoglycan - Domain-Structure of the Receptor.* Journal of Biological Chemistry, 1988. **263**(32): p. 16984-16991.
728. Cheifetz, S., et al., *Endoglin Is a Component of the Transforming Growth-Factor-Beta Receptor System in Human Endothelial-Cells.* Journal of Biological Chemistry, 1992. **267**(27): p. 19027-19030.
729. Nassiri, F., et al., *Endoglin (CD105): A Review of its Role in Angiogenesis and Tumor Diagnosis, Progression and Therapy.* Anticancer Research, 2011. **31**(6): p. 2283-2290.
730. Esparza-Lopez, J., et al., *Ligand binding and functional properties of betaglycan, a co-receptor of the transforming growth factor-beta superfamily - Specialized binding*

regions for transforming growth factor-beta and inhibin A. Journal of Biological Chemistry, 2001. **276**(18): p. 14588-14596.

731. Lopezcasillas, F., J.L. Wrana, and J. Massague, *Betaglycan Presents Ligand to the Tgf-Beta Signaling Receptor.* Cell, 1993. **73**(7): p. 1435-1444.
732. Yamashita, H., et al., *Endoglin Forms a Heteromeric Complex with the Signaling Receptors for Transforming Growth-Factor-Beta.* Journal of Biological Chemistry, 1994. **269**(3): p. 1995-2001.
733. Khalil, N., *TGF-beta: from latent to active.* Microbes and Infection, 1999. **1**(15): p. 1255-1263.
734. Gentry, L.E. and B.W. Nash, *The Pro Domain of Pre-Pro-Transforming Growth Factor-Beta-1 When Independently Expressed Is a Functional Binding-Protein for the Mature Growth-Factor.* Biochemistry, 1990. **29**(29): p. 6851-6857.
735. Munger, J.S., et al., *Latent transforming growth factor-beta: Structural features and mechanisms of activation.* Kidney International, 1997. **51**(5): p. 1376-1382.
736. Lawrence, D.A., *Transforming growth factor-beta: A general review.* European Cytokine Network, 1996. **7**(3): p. 363-374.
737. Reuning, U., et al., *Multifunctional potential of the plasminogen activation system in tumor invasion and metastasis (Review).* International Journal of Oncology, 1998. **13**(5): p. 893-906.
738. Sato, Y., et al., *Characterization of the Activation of Latent Tgf-Beta by Cocultures of Endothelial-Cells and Pericytes or Smooth-Muscle Cells - a Self-Regulating System.* Journal of Cell Biology, 1990. **111**(2): p. 757-763.
739. Miyazono, K., H. Ichijo, and C.H. Heldin, *Transforming Growth-Factor-Beta - Latent Forms, Binding-Proteins and Receptors.* Growth Factors, 1993. **8**(1): p. 11-22.
740. Gressner, O.A., R. Weiskirchen, and A.M. Gressner, *Evolving concepts of liver fibrogenesis provide new diagnostic and therapeutic options.* Comp Hepatol, 2007. **6**: p. 7.
741. Sun, P.D. and D.R. Davies, *The Cystine-Knot Growth-Factor Superfamily.* Annual Review of Biophysics and Biomolecular Structure, 1995. **24**: p. 269-291.
742. Shi, Y.G. and J. Massague, *Mechanisms of TGF-beta signaling from cell membrane to the nucleus.* Cell, 2003. **113**(6): p. 685-700.
743. Rubtsov, Y.P. and A.Y. Rudensky, *TGFbeta signalling in control of T-cell-mediated self-reactivity.* Nat Rev Immunol, 2007. **7**(6): p. 443-53.
744. Derynck, R. and Y.E. Zhang, *Smad-dependent and Smad-independent pathways in TGF-beta family signalling.* Nature, 2003. **425**(6958): p. 577-584.
745. Zhang, Y., et al., *Receptor-associated Mad homologues synergize as effectors of the TGF-beta response.* Nature, 1996. **383**(6596): p. 168-172.
746. Nakao, A., et al., *TGF-beta receptor-mediated signalling through Smad2, Smad3 and Smad4.* Embo Journal, 1997. **16**(17): p. 5353-5362.
747. Itoh, F., et al., *The FYVE domain in Smad anchor for receptor activation (SARA) is sufficient for localization of SARA in early endosomes and regulates TGF-beta/Smad signalling.* Genes to Cells, 2002. **7**(3): p. 321-331.
748. Liu, L., et al., *Smad2 and Smad3 have differential sensitivity in relaying TGF beta signaling and inversely regulate early lineage specification.* Scientific Reports, 2016. **6**.
749. Schmierer, B. and C.S. Hill, *TGF beta-SMAD signal transduction: molecular specificity and functional flexibility.* Nature Reviews Molecular Cell Biology, 2007. **8**(12): p. 970-982.
750. Zi, Z.K., D.A. Chapnick, and X.D. Liu, *Dynamics of TGF-beta/Smad signaling.* Febs Letters, 2012. **586**(14): p. 1921-1928.

751. Zhang, Y.E., *Non-Smad pathways in TGF-beta signaling.* Cell Research, 2009. **19**(1): p. 128-139.
752. Mulder, K.M. and S.L. Morris, *Activation of P21ras by Transforming Growth-Factor-Beta in Epithelial-Cells.* Journal of Biological Chemistry, 1992. **267**(8): p. 5029-5031.
753. Hartsough, M.T. and K.M. Mulder, *Transforming Growth-Factor-Beta Activation of P44(Mapk) in Proliferating Cultures of Epithelial-Cells.* Journal of Biological Chemistry, 1995. **270**(13): p. 7117-7124.
754. Frey, R.S. and K.M. Mulder, *TGFbeta regulation of mitogen-activated protein kinases in human breast cancer cells.* Cancer Lett, 1997. **117**(1): p. 41-50.
755. Mucsi, I., K.L. Skorecki, and H.J. Goldberg, *Extracellular signal-regulated kinase and the small GTP-binding protein, Rac, contribute to the effects of transforming growth factor-beta1 on gene expression.* J Biol Chem, 1996. **271**(28): p. 16567-72.
756. Olsson, N., et al., *Transforming growth factor-beta-mediated mast cell migration depends on mitogen-activated protein kinase activity.* Cell Signal, 2001. **13**(7): p. 483-90.
757. Galliher, A.J. and W.P. Schiemann, *Src phosphorylates Tyr284 in TGF-beta type II receptor and regulates TGF-beta stimulation of p38 MAPK during breast cancer cell proliferation and invasion.* Cancer Res, 2007. **67**(8): p. 3752-8.
758. Lee, M.K., et al., *TGF-beta activates Erk MAP kinase signalling through direct phosphorylation of ShcA.* Embo Journal, 2007. **26**(17): p. 3957-3967.
759. Davies, M., et al., *Induction of an epithelial to mesenchymal transition in human immortal and malignant keratinocytes by TGF-beta1 involves MAPK, Smad and AP-1 signalling pathways.* J Cell Biochem, 2005. **95**(5): p. 918-31.
760. Zavadil, J., et al., *Genetic programs of epithelial cell plasticity directed by transforming growth factor-beta.* Proc Natl Acad Sci U S A, 2001. **98**(12): p. 6686-91.
761. Galliher-Beckley, A.J. and W.P. Schiemann, *Grb2 binding to Tyr284 in T beta R-II is essential for mammary tumor growth and metastasis stimulated by TGF-beta.* Carcinogenesis, 2008. **29**(2): p. 244-251.
762. Matsuura, I., et al., *Identification and characterization of ERK MAP kinase phosphorylation sites in Smad3.* Biochemistry, 2005. **44**(37): p. 12546-12553.
763. Hocevar, B.A., T.L. Brown, and P.H. Howe, *TGF-beta induces fibronectin synthesis through a c-Jun N-terminal kinase-dependent, Smad4-independent pathway.* Embo Journal, 1999. **18**(5): p. 1345-1356.
764. Yamaguchi, K., et al., *Identification of a Member of the Mapkkk Family as a Potential Mediator of Tgf-Beta Signal-Transduction.* Science, 1995. **270**(5244): p. 2008-2011.
765. Shim, J.H., et al., *TAK1, but not TAB1 or TAB2, plays an essential role in multiple signaling pathways in vivo.* Genes & Development, 2005. **19**(22): p. 2668-2681.
766. Sorrentino, A., et al., *The type I TGF-beta receptor engages TRAF6 to activate TAK1 in a receptor kinase-independent manner.* Nature Cell Biology, 2008. **10**(10): p. 1199-1207.
767. Yamashita, M., et al., *TRAF6 mediates Smad-independent activation of JNK and p38 by TGF-beta.* Molecular Cell, 2008. **31**(6): p. 918-924.
768. Atfi, A., et al., *Evidence for a role of Rho-like GTPases and stress-activated protein kinase/c-Jun N-terminal kinase (SAPK/JNK) in transforming growth factor beta-mediated signaling.* Journal of Biological Chemistry, 1997. **272**(3): p. 1429-1432.
769. Kimura, N., et al., *BMP2-induced apoptosis is mediated by activation of the TAK1-p38 kinase pathway that is negatively regulated by Smad6.* Journal of Biological Chemistry, 2000. **275**(23): p. 17647-17652.
770. Bakin, A.V., et al., *p38 mitogen-activated protein kinase is required for TGF beta-mediated fibroblastic transdifferentiation and cell migration.* Journal of Cell Science, 2002. **115**(15): p. 3193-3206.

771. Bhowmick, N.A., et al., *Transforming growth factor-beta 1 mediates epithelial to mesenchymal transdifferentiation through a RhoA-dependent mechanism.* Molecular Biology of the Cell, 2001. **12**(1): p. 27-36.
772. Edlund, S., et al., *Transforming growth factor-beta-induced mobilization of actin cytoskeleton requires signaling by small GTPases Cdc42 and RhoA.* Molecular Biology of the Cell, 2002. **13**(3): p. 902-914.
773. Shen, X., et al., *The activity of guanine exchange factor NET1 is essential for transforming growth factor-beta-mediated stress fiber formation.* Journal of Biological Chemistry, 2001. **276**(18): p. 15362-15368.
774. Ozdamar, B., et al., *Regulation of the polarity protein Par6 by TGF beta receptors controls epithelial cell plasticity.* Science, 2005. **307**(5715): p. 1603-1609.
775. Wang, H.R., et al., *Regulation of cell polarity and protrusion formation by targeting RhoA for degradation.* Science, 2003. **302**(5651): p. 1775-1779.
776. Barrios-Rodiles, M., et al., *High-throughput mapping of a dynamic signaling network in mammalian cells.* Science, 2005. **307**(5715): p. 1621-1625.
777. Edlund, S., et al., *Smad7 is required for TGF-beta-induced activation of the small GTPase Cdc42.* Journal of Cell Science, 2004. **117**(9): p. 1835-1846.
778. Yi, J.Y., I. Shin, and C.L. Arteaga, *Type I transforming growth factor beta receptor binds to and activates phosphatidylinositol 3-kinase.* Journal of Biological Chemistry, 2005. **280**(11): p. 10870-10876.
779. Valderrama-Carvajal, H., et al., *Activin/TGF-beta induce apoptosis through Smad-dependent expression of the lipid phosphatase SHIP.* Nature Cell Biology, 2002. **4**(12): p. 963-969.
780. Lamouille, S. and R. Derynck, *Cell size and invasion in TGF-beta-induced epithelial to mesenchymal transition is regulated by activation of the mTOR pathway.* Journal of Cell Biology, 2007. **178**(3): p. 437-451.
781. Wang, S.N., et al., *Imatinib mesylate blocks a non-smad TGF-beta pathway and reduces renal fibrogenesis in vivo.* Faseb Journal, 2005. **19**(1): p. 1-11.
782. Conery, A.R., et al., *Akt interacts directly with Smad3 to regulate the sensitivity to TGF-beta-induced apoptosis.* Nature Cell Biology, 2004. **6**(4): p. 366-372.
783. Remy, I., A. Montmarquette, and S.W. Michnick, *PKB/Akt modulates TGF-beta signalling through a direct interaction with Smad3.* Nature Cell Biology, 2004. **6**(4): p. 358-365.
784. Petritsch, C., et al., *TGF-beta inhibits p70 S6 kinase via protein phosphatase 2A to induce G(1) arrest.* Genes & Development, 2000. **14**(24): p. 3093-3101.
785. Papageorgis, P. and T. Stylianopoulos, *Role of TGFbeta in regulation of the tumor microenvironment and drug delivery (review).* Int J Oncol, 2015. **46**(3): p. 933-43.
786. Yue, J.B. and K.M. Mulder, *Requirement of Ras/MAPK pathway activation by transforming growth factor beta for transforming growth Factor beta(1) production in a Smad-dependent pathway. (vol 275, pg 30765, 2000).* Journal of Biological Chemistry, 2000. **275**(45): p. 35656-35656.
787. Gotzmann, J., et al., *A crucial function of PDGF in TGF-beta-mediated cancer progression of hepatocytes.* Oncogene, 2006. **25**(22): p. 3170-3185.
788. Guo, X. and X.F. Wang, *Signaling cross-talk between TGF-beta/BMP and other pathways.* Cell Research, 2009. **19**(1): p. 71-88.
789. Dennler, S., et al., *Induction of sonic hedgehog mediators by transforming growth factor-beta: Smad3-dependent activation of Gli2 and Gli1 expression in vitro and in vivo.* Cancer Research, 2007. **67**(14): p. 6981-6986.
790. Alvarez-Rodiguez, R., et al., *Bone morphogenetic protein 2 opposes Shh-mediated proliferation in cerebellar granule cells through a TIEG-1-based regulation of Nmyc.* Journal of Biological Chemistry, 2007. **282**(51): p. 37170-37180.

791. Massague, J., *TGF beta signalling in context.* Nature Reviews Molecular Cell Biology, 2012. **13**(10): p. 616-630.
792. Heldin, C.H., M. Landstrom, and A. Moustakas, *Mechanism of TGF-beta signaling to growth arrest, apoptosis, and epithelial-mesenchymal transition.* Current Opinion in Cell Biology, 2009. **21**(2): p. 166-176.
793. Gomis, R.R., et al., *A FoxO-Smad synexpression group in human keratinocytes.* Proceedings of the National Academy of Sciences of the United States of America, 2006. **103**(34): p. 12747-12752.
794. Orkin, S.H. and K. Hochedlinger, *Chromatin Connections to Pluripotency and Cellular Reprogramming.* Cell, 2011. **145**(6): p. 835-850.
795. Mullen, A.C., et al., *Master Transcription Factors Determine Cell-Type-Specific Responses to TGF-beta Signaling.* Cell, 2011. **147**(3): p. 565-576.
796. Blahna, M.T. and A. Hata, *Regulation of miRNA biogenesis as an integrated component of growth factor signaling.* Current Opinion in Cell Biology, 2013. **25**(2): p. 233-240.
797. Hannon, G.J. and D. Beach, *P15(Ink4b) Is a Potential Effector of Tgf-Beta-Induced Cell-Cycle Arrest.* Nature, 1994. **371**(6494): p. 257-261.
798. Polyak, K., et al., *P27(Kip1), a Cyclin-Cdk Inhibitor, Links Transforming Growth-Factor-Beta and Contact Inhibition to Cell-Cycle Arrest.* Genes & Development, 1994. **8**(1): p. 9-22.
799. Datto, M.B., et al., *Transforming Growth-Factor-Beta Induces the Cyclin-Dependent Kinase Inhibitor P21 through a P53-Independent Mechanism.* Proceedings of the National Academy of Sciences of the United States of America, 1995. **92**(12): p. 5545-5549.
800. Pietenpol, J.A., et al., *Tgf-Beta-1 Inhibition of C-Myc Transcription and Growth in Keratinocytes Is Abrogated by Viral Transforming Proteins with Prb Binding Domains.* Cell, 1990. **61**(5): p. 777-785.
801. Iavarone, A. and J. Massague, *Repression of the CDK activator Cdc25A and cell-cycle arrest by cytokine TGF-beta in cells lacking the CDK inhibitor p15.* Nature, 1997. **387**(6631): p. 417-422.
802. Jian, H.Y., et al., *Smad3-dependent nuclear translocation of beta-catenin is required for TGF-beta 1-induced proliferation of bone marrow-derived adult human mesenchymal stem cells.* Genes & Development, 2006. **20**(6): p. 666-674.
803. Bruna, A., et al., *High TGF beta-Smad activity confers poor prognosis in glioma patients and promotes cell proliferation depending on the methylation of the PDGF-B gene.* Cancer Cell, 2007. **11**(2): p. 147-160.
804. Li, M.O. and R.A. Flavell, *TGF-beta: A master of all T cell trades.* Cell, 2008. **134**(3): p. 392-404.
805. Blank, U. and S. Karlsson, *TGF-beta signaling in the control of hematopoietic stem cells.* Blood, 2015. **125**(23): p. 3542-3550.
806. Krieglstein, K., et al., *More than being protective: functional roles for TGF-beta/activin signaling pathways at central synapses.* Trends in Neurosciences, 2011. **34**(8): p. 421-429.
807. Vogel, T., et al., *Transforming growth factor beta promotes neuronal cell fate of mouse cortical and hippocampal progenitors in vitro and in vivo: identification of Nedd9 as an essential signaling component.* Cereb Cortex, 2010. **20**(3): p. 661-71.
808. Kaartinen, V., et al., *Abnormal lung development and cleft palate in mice lacking TGF-beta 3 indicates defects of epithelial-mesenchymal interaction.* Nat Genet, 1995. **11**(4): p. 415-21.

809. Shull, M.M., et al., *Targeted disruption of the mouse transforming growth factor-beta 1 gene results in multifocal inflammatory disease.* Nature, 1992. **359**(6397): p. 693-9.
810. Ignotz, R.A. and J. Massague, *Type beta transforming growth factor controls the adipogenic differentiation of 3T3 fibroblasts.* Proc Natl Acad Sci U S A, 1985. **82**(24): p. 8530-4.
811. Seyedin, S.M., et al., *Purification and characterization of two cartilage-inducing factors from bovine demineralized bone.* Proc Natl Acad Sci U S A, 1985. **82**(8): p. 2267-71.
812. Hassani, S.N., et al., *Simultaneous suppression of TGF-beta and ERK signaling contributes to the highly efficient and reproducible generation of mouse embryonic stem cells from previously considered refractory and non-permissive strains.* Stem Cell Rev, 2012. **8**(2): p. 472-81.
813. Sporn, M.B., et al., *Polypeptide transforming growth factors isolated from bovine sources and used for wound healing in vivo.* Science, 1983. **219**(4590): p. 1329-31.
814. Wahl, S.M., et al., *Transforming growth factor type beta induces monocyte chemotaxis and growth factor production.* Proc Natl Acad Sci U S A, 1987. **84**(16): p. 5788-92.
815. Postlethwaite, A.E., et al., *Stimulation of the chemotactic migration of human fibroblasts by transforming growth factor beta.* J Exp Med, 1987. **165**(1): p. 251-6.
816. Edwards, D.R., et al., *Transforming growth factor beta modulates the expression of collagenase and metalloproteinase inhibitor.* EMBO J, 1987. **6**(7): p. 1899-904.
817. Kalluri, R. and R.A. Weinberg, *The basics of epithelial-mesenchymal transition.* J Clin Invest, 2009. **119**(6): p. 1420-8.
818. Ashcroft, G.S., et al., *Mice lacking Smad3 show accelerated wound healing and an impaired local inflammatory response.* Nat Cell Biol, 1999. **1**(5): p. 260-6.
819. de Martin, R., et al., *Complementary DNA for human glioblastoma-derived T cell suppressor factor, a novel member of the transforming growth factor-beta gene family.* EMBO J, 1987. **6**(12): p. 3673-7.
820. Veldhoen, M., et al., *TGFbeta in the context of an inflammatory cytokine milieu supports de novo differentiation of IL-17-producing T cells.* Immunity, 2006. **24**(2): p. 179-89.
821. Miyazono, K., *Positive and negative regulation of TGF-beta signaling.* J Cell Sci, 2000. **113 (Pt 7)**: p. 1101-9.
822. de Caestecker, M.P., et al., *Smad2 transduces common signals from receptor serine-threonine and tyrosine kinases.* Genes Dev, 1998. **12**(11): p. 1587-92.
823. Zhang, Y., X.H. Feng, and R. Derynck, *Smad3 and Smad4 cooperate with c-Jun/c-Fos to mediate TGF-beta-induced transcription.* Nature, 1998. **394**(6696): p. 909-13.
824. Zimmerman, L.B., J.M. De Jesus-Escobar, and R.M. Harland, *The Spemann organizer signal noggin binds and inactivates bone morphogenetic protein 4.* Cell, 1996. **86**(4): p. 599-606.
825. Thisse, C. and B. Thisse, *Antivin, a novel and divergent member of the TGFbeta superfamily, negatively regulates mesoderm induction.* Development, 1999. **126**(2): p. 229-40.
826. Onichtchouk, D., et al., *Silencing of TGF-beta signalling by the pseudoreceptor BAMBI.* Nature, 1999. **401**(6752): p. 480-5.
827. Chen, Y.G., F. Liu, and J. Massague, *Mechanism of TGFbeta receptor inhibition by FKBP12.* EMBO J, 1997. **16**(13): p. 3866-76.
828. Nagarajan, R.P., et al., *Regulation of Smad7 promoter by direct association with Smad3 and Smad4.* J Biol Chem, 1999. **274**(47): p. 33412-8.

829. Kretzschmar, M., J. Doody, and J. Massague, *Opposing BMP and EGF signalling pathways converge on the TGF-beta family mediator Smad1.* Nature, 1997. **389**(6651): p. 618-22.
830. Kretzschmar, M., et al., *A mechanism of repression of TGFbeta/ Smad signaling by oncogenic Ras.* Genes Dev, 1999. **13**(7): p. 804-16.
831. Wotton, D., et al., *A Smad transcriptional corepressor.* Cell, 1999. **97**(1): p. 29-39.
832. Luo, K., et al., *The Ski oncoprotein interacts with the Smad proteins to repress TGFbeta signaling.* Genes Dev, 1999. **13**(17): p. 2196-206.
833. Zhu, H., et al., *A SMAD ubiquitin ligase targets the BMP pathway and affects embryonic pattern formation.* Nature, 1999. **400**(6745): p. 687-93.
834. Vivien, D. and C. Ali, *Transforming growth factor-beta signalling in brain disorders.* Cytokine Growth Factor Rev, 2006. **17**(1-2): p. 121-8.
835. Vivien, D., et al., *Evidence of type I and type II transforming growth factor-beta receptors in central nervous tissues: changes induced by focal cerebral ischemia.* J Neurochem, 1998. **70**(6): p. 2296-304.
836. Kano, K., et al., *Cloning and studies of the mouse cDNA encoding Smad3.* J Vet Med Sci, 1999. **61**(3): p. 213-9.
837. da Cunha, A., et al., *Glial cell-specific mechanisms of TGF-beta 1 induction by IL-1 in cerebral cortex.* J Neuroimmunol, 1993. **42**(1): p. 71-85.
838. Dobolyi, A., et al., *The neuroprotective functions of transforming growth factor beta proteins.* Int J Mol Sci, 2012. **13**(7): p. 8219-58.
839. Mathieu, P., A.P. Piantanida, and F. Pitossi, *Chronic expression of transforming growth factor-beta enhances adult neurogenesis.* Neuroimmunomodulation, 2010. **17**(3): p. 200-1.
840. Heupel, K., et al., *Loss of transforming growth factor-beta 2 leads to impairment of central synapse function.* Neural Dev, 2008. **3**: p. 25.
841. Prevot, V., et al., *Evidence that members of the TGFbeta superfamily play a role in regulation of the GnRH neuroendocrine axis: expression of a type I serine-threonine kinase receptor for TGRbeta and activin in GnRH neurones and hypothalamic areas of the female rat.* J Neuroendocrinol, 2000. **12**(7): p. 665-70.
842. Fevre-Montange, M., et al., *Localization of transforming growth factors, TGFbeta1 and TGFbeta3, in hypothalamic magnocellular neurones and the neurohypophysis.* J Neuroendocrinol, 2004. **16**(7): p. 571-6.
843. Kiefer, R., et al., *Transforming growth factor-beta 1: a lesion-associated cytokine of the nervous system.* Int J Dev Neurosci, 1995. **13**(3-4): p. 331-9.
844. Yu, P., et al., *An in vitro model of reactive astrogliosis and its effect on neuronal growth.* Methods Mol Biol, 2012. **814**: p. 327-40.
845. Bierie, B. and H.L. Moses, *Tumour microenvironment: TGFbeta: the molecular Jekyll and Hyde of cancer.* Nat Rev Cancer, 2006. **6**(7): p. 506-20.
846. Massague, J., *TGFbeta in Cancer.* Cell, 2008. **134**(2): p. 215-30.
847. Levy, L. and C.S. Hill, *Alterations in components of the TGF-beta superfamily signaling pathways in human cancer.* Cytokine Growth Factor Rev, 2006. **17**(1-2): p. 41-58.
848. Sjoblom, T., et al., *The consensus coding sequences of human breast and colorectal cancers.* Science, 2006. **314**(5797): p. 268-74.
849. Jaffee, E.M., et al., *Focus on pancreas cancer.* Cancer Cell, 2002. **2**(1): p. 25-8.
850. Cerutti, J.M., et al., *Expression of Smad4 and Smad7 in human thyroid follicular carcinoma cell lines.* J Endocrinol Invest, 2003. **26**(6): p. 516-21.
851. Dowdy, S.C., et al., *Overexpression of the TGF-beta antagonist Smad7 in endometrial cancer.* Gynecol Oncol, 2005. **96**(2): p. 368-73.

852. Drummond, A. and J. Findlay, *Focus on TGF-beta signalling.* Reproduction, 2006. **132**(2): p. 177-8.
853. Chen, C.R., Y. Kang, and J. Massague, *Defective repression of c-myc in breast cancer cells: A loss at the core of the transforming growth factor beta growth arrest program.* Proc Natl Acad Sci U S A, 2001. **98**(3): p. 992-9.
854. Sanchez-Capelo, A., *Dual role for TGF-beta1 in apoptosis.* Cytokine Growth Factor Rev, 2005. **16**(1): p. 15-34.
855. Gordon, K.J. and G.C. Blobe, *Role of transforming growth factor-beta superfamily signaling pathways in human disease.* Biochim Biophys Acta, 2008. **1782**(4): p. 197-228.
856. Chen, T., et al., *Structural alterations of transforming growth factor-beta receptor genes in human cervical carcinoma.* Int J Cancer, 1999. **82**(1): p. 43-51.
857. Myeroff, L.L., et al., *A transforming growth factor beta receptor type II gene mutation common in colon and gastric but rare in endometrial cancers with microsatellite instability.* Cancer Res, 1995. **55**(23): p. 5545-7.
858. Halder, S.K., R.D. Beauchamp, and P.K. Datta, *Smad7 induces tumorigenicity by blocking TGF-beta-induced growth inhibition and apoptosis.* Exp Cell Res, 2005. **307**(1): p. 231-46.
859. Kuang, C., et al., *In vivo disruption of TGF-beta signaling by Smad7 leads to premalignant ductal lesions in the pancreas.* Proc Natl Acad Sci U S A, 2006. **103**(6): p. 1858-63.
860. Conery, A.R., et al., *Akt interacts directly with Smad3 to regulate the sensitivity to TGF-beta induced apoptosis.* Nat Cell Biol, 2004. **6**(4): p. 366-72.
861. Nagaraj, N.S. and P.K. Datta, *Targeting the transforming growth factor-beta signaling pathway in human cancer.* Expert Opin Investig Drugs, 2010. **19**(1): p. 77-91.
862. Dooley, S., et al., *Hepatocyte-specific Smad7 expression attenuates TGF-beta-mediated fibrogenesis and protects against liver damage.* Gastroenterology, 2008. **135**(2): p. 642-59.
863. Derynck, R. and R.J. Akhurst, *Differentiation plasticity regulated by TGF-beta family proteins in development and disease.* Nat Cell Biol, 2007. **9**(9): p. 1000-4.
864. Valcourt, U., et al., *TGF-beta and the Smad signaling pathway support transcriptomic reprogramming during epithelial-mesenchymal cell transition.* Mol Biol Cell, 2005. **16**(4): p. 1987-2002.
865. Mani, S.A., et al., *The epithelial-mesenchymal transition generates cells with properties of stem cells.* Cell, 2008. **133**(4): p. 704-15.
866. Hau, P., et al., *Inhibition of TGF-beta2 with AP 12009 in recurrent malignant gliomas: from preclinical to phase I/II studies.* Oligonucleotides, 2007. **17**(2): p. 201-12.
867. Nemunaitis, J., et al., *Phase II study of belagenpumatucel-L, a transforming growth factor beta-2 antisense gene-modified allogeneic tumor cell vaccine in non-small-cell lung cancer.* J Clin Oncol, 2006. **24**(29): p. 4721-30.
868. Morris, J.C., et al., *Phase I study of GC1008 (fresolimumab): a human anti-transforming growth factor-beta (TGFbeta) monoclonal antibody in patients with advanced malignant melanoma or renal cell carcinoma.* PLoS One, 2014. **9**(3): p. e90353.
869. Smith, A.L., T.P. Robin, and H.L. Ford, *Molecular pathways: targeting the TGF-beta pathway for cancer therapy.* Clin Cancer Res, 2012. **18**(17): p. 4514-21.
870. Herbertz, S., et al., *Clinical development of galunisertib (LY2157299 monohydrate), a small molecule inhibitor of transforming growth factor-beta signaling pathway.* Drug Des Devel Ther, 2015. **9**: p. 4479-99.

871. Neuzillet, C., et al., *Targeting the TGFbeta pathway for cancer therapy.* Pharmacol Ther, 2015. **147**: p. 22-31.
872. Quail, D.F. and J.A. Joyce, *Microenvironmental regulation of tumor progression and metastasis.* Nature Medicine, 2013. **19**(11): p. 1423-1437.
873. Katt, M.E., et al., *In Vitro Tumor Models: Advantages, Disadvantages, Variables, and Selecting the Right Platform.* Front Bioeng Biotechnol, 2016. **4**: p. 12.
874. Joeckel, E., et al., *High calcium concentration in bones promotes bone metastasis in renal cell carcinomas expressing calcium-sensing receptor.* Molecular Cancer, 2014. **13**.
875. Friedl, P., et al., *New dimensions in cell migration.* Nature Reviews Molecular Cell Biology, 2012. **13**(11): p. 743-747.
876. Hakkinen, K.M., et al., *Direct Comparisons of the Morphology, Migration, Cell Adhesions, and Actin Cytoskeleton of Fibroblasts in Four Different Three-Dimensional Extracellular Matrices.* Tissue Engineering Part A, 2011. **17**(5-6): p. 713-724.
877. Liang, Y., et al., *Type I collagen is overexpressed in medulloblastoma as a component of tumor microenvironment.* Journal of Neuro-Oncology, 2008. **86**(2): p. 133-141.
878. Vernon, R.B. and M.D. Gooden, *New technologies in vitro for analysis of cell movement on or within collagen gels.* Matrix Biology, 2002. **21**(8): p. 661-669.
879. Yarrow, J.C., et al., *Screening for cell migration inhibitors via automated microscopy reveals a rho-kinase inhibitor.* Chemistry & Biology, 2005. **12**(3): p. 385-395.
880. Raeber, G.P., M.P. Lutolf, and J.A. Hubbell, *Molecularly engineered PEG hydrogels: A novel model system for proteolytically mediated cell migration.* Biophysical Journal, 2005. **89**(2): p. 1374-1388.
881. Evensen, N.A., et al., *Development of a High-Throughput Three-Dimensional Invasion Assay for Anti-Cancer Drug Discovery.* Plos One, 2013. **8**(12).
882. Onvani, S., et al., *Molecular genetic analysis of the hepatocyte growth factor/MET signaling pathway in pediatric medulloblastoma.* Genes Chromosomes & Cancer, 2012. **51**(7): p. 675-688.
883. Katayama, R., et al., *Cytotoxic activity of tivantinib (ARQ 197) is not due solely to c-MET inhibition.* Cancer Res, 2013. **73**(10): p. 3087-96.
884. Sequist, L.V., et al., *Randomized phase II study of erlotinib plus tivantinib versus erlotinib plus placebo in previously treated non-small-cell lung cancer.* J Clin Oncol, 2011. **29**(24): p. 3307-15.
885. Abidoye, O., N. Murukurthy, and R. Salgia, *Review of clinic trials: agents targeting c-Met.* Rev Recent Clin Trials, 2007. **2**(2): p. 143-7.
886. Adjei, A.A., B. Schwartz, and E. Garmey, *Early clinical development of ARQ 197, a selective, non-ATP-competitive inhibitor targeting MET tyrosine kinase for the treatment of advanced cancers.* Oncologist, 2011. **16**(6): p. 788-99.
887. Buonamici, S., et al., *Interfering with resistance to smoothened antagonists by inhibition of the PI3K pathway in medulloblastoma.* Sci Transl Med, 2010. **2**(51): p. 51ra70.
888. Yan, W., et al., *The Nck-interacting kinase (NIK) phosphorylates the Na+-H+ exchanger NHE1 and regulates NHE1 activation by platelet-derived growth factor.* J Biol Chem, 2001. **276**(33): p. 31349-56.
889. Yao, Z., et al., *A novel human STE20-related protein kinase, HGK, that specifically activates the c-Jun N-terminal kinase signaling pathway.* J Biol Chem, 1999. **274**(4): p. 2118-25.
890. Dudu, V., et al., *Role of Epidermal Growth Factor-Triggered PI3K/Akt Signaling in the Migration of Medulloblastoma-Derived Cells.* Cell Mol Bioeng, 2012. **5**(4): p. 502-413.

891. Jakacki, R.I., et al., *Pediatric phase I and pharmacokinetic study of erlotinib followed by the combination of erlotinib and temozolomide: a Children's Oncology Group Phase I Consortium Study.* J Clin Oncol, 2008. **26**(30): p. 4921-7.
892. Wang, D., et al., *Clinical experience of MEK inhibitors in cancer therapy.* Biochim Biophys Acta, 2007. **1773**(8): p. 1248-55.
893. Wong, D.J., et al., *Erratum to: Antitumor activity of the ERK inhibitor SCH722984 against BRAF mutant, NRAS mutant and wild-type melanoma.* Mol Cancer, 2015. **14**: p. 128.
894. Radu, M., et al., *PAK signalling during the development and progression of cancer.* Nat Rev Cancer, 2014. **14**(1): p. 13-25.
895. Dennler, S., et al., *Induction of sonic hedgehog mediators by transforming growth factor-beta: Smad3-dependent activation of Gli2 and Gli1 expression in vitro and in vivo.* Cancer Res, 2007. **67**(14): p. 6981-6.
896. Aref, D., et al., *Canonical TGF-beta pathway activity is a predictor of SHH-driven medulloblastoma survival and delineates putative precursors in cerebellar development.* Brain Pathol, 2013. **23**(2): p. 178-91.
897. Gate, D., et al., *T-cell TGF-beta signaling abrogation restricts medulloblastoma progression.* Proc Natl Acad Sci U S A, 2014. **111**(33): p. E3458-66.
898. Wei, L., et al., *Novel Insights into the Roles of Rho Kinase in Cancer.* Arch Immunol Ther Exp (Warsz), 2016. **64**(4): p. 259-78.
899. Liu, S., et al., *Inhibition of rho-associated kinase signaling prevents breast cancer metastasis to human bone.* Cancer Res, 2009. **69**(22): p. 8742-51.
900. Jeong, K.J., et al., *The Rho/ROCK pathway for lysophosphatidic acid-induced proteolytic enzyme expression and ovarian cancer cell invasion.* Oncogene, 2012. **31**(39): p. 4279-89.
901. Rana, M.K. and R.A. Worthylake, *Novel mechanism for negatively regulating Rho-kinase (ROCK) signaling through Coronin1B protein in neuregulin 1 (NRG-1)-induced tumor cell motility.* J Biol Chem, 2012. **287**(26): p. 21836-45.
902. Vishnubhotla, R., et al., *Treatment with Y-27632, a ROCK Inhibitor, Increases the Proinvasive Nature of SW620 Cells on 3D Collagen Type 1 Matrix.* Int J Cell Biol, 2012. **2012**: p. 259142.
903. Budhu, A., et al., *Prediction of venous metastases, recurrence, and prognosis in hepatocellular carcinoma based on a unique immune response signature of the liver microenvironment.* Cancer Cell, 2006. **10**(2): p. 99-111.
904. Sharma, M., et al., *Analysis of stromal signatures in the tumor microenvironment of ductal carcinoma in situ.* Breast Cancer Res Treat, 2010. **123**(2): p. 397-404.
905. Emmenegger, B.A., et al., *Distinct roles for fibroblast growth factor signaling in cerebellar development and medulloblastoma.* Oncogene, 2013. **32**(35): p. 4181-8.
906. Thoma, C.R., et al., *3D cell culture systems modeling tumor growth determinants in cancer target discovery.* Adv Drug Deliv Rev, 2014. **69-70**: p. 29-41.
907. Connolly, E.C., J. Freimuth, and R.J. Akhurst, *Complexities of TGF-beta targeted cancer therapy.* Int J Biol Sci, 2012. **8**(7): p. 964-78.
908. Matsuoka, T., et al., *RhoA/ROCK signaling mediates plasticity of scirrhous gastric carcinoma motility.* Clin Exp Metastasis, 2011. **28**(7): p. 627-36.
909. Matsuoka, T. and M. Yashiro, *Rho/ROCK signaling in motility and metastasis of gastric cancer.* World J Gastroenterol, 2014. **20**(38): p. 13756-66.
910. Kale, V.P., et al., *A novel selective multikinase inhibitor of ROCK and MRCK effectively blocks cancer cell migration and invasion.* Cancer Lett, 2014. **354**(2): p. 299-310.
911. Alexander, P.B. and X.F. Wang, *Resistance to receptor tyrosine kinase inhibition in cancer: molecular mechanisms and therapeutic strategies.* Front Med, 2015. **9**(2): p. 134-8.

912. Ajeawung, N.F., H.Y. Wang, and D. Kamnasaran, *Progress from clinical trials and emerging non-conventional therapies for the treatment of Medulloblastomas.* Cancer Letters, 2013. **330**(2): p. 130-140.

7. List of Abbreviations

- Abbreviations used

7. List of abbreviations

4EBP	-	4E-binding protein-1
aa	-	Amino acids
ACCIS	-	Automated Childhood Cancer Information System
aCDc	-	Automated Cell Dissemination Counter
ADAMTS	-	Metalloproteinase with thrombospondin motifs
TSP-1	-	Thromobospondin type-1
ADP	-	Adenosine diphosphate
AKT	-	Protein kinase B
ALK	-	Anaplastic lymphoma kinase
ARE	-	Activin response elements
Arp2/3	-	Actin nucleation complex
ATP	-	Adenosine triphosphate
BBB	-	Blood-brain barrier
BCL-2	-	B-cell lymphoma 2
BCR	-	Breakpoint cluster region
BDNF	-	Brain derived neurotrophic factor
bFGF	-	basic fibroblast growth factor
BM	-	Basement membrane
BMPs	-	Bone morphogenic proteins
BTSCs	-	Brain tumour stem cells
CBTRUS	-	Central Brain Tumour Registry of the USA
CCNU	-	1-(2-chloro-ethyl)-3-cyclohexyl-1-nitrosourea
CCSS	-	Childhood Cancer Survivor Study
CD68	-	Cluster of differentiation 68
Cdc42	-	Cell division control protein 42 homolog
Cdks	-	Cyclin-dependent kinases
CECT	-	Contrast enhanced computed tomography
CNH	-	Citron homology domain
CNS	-	Central nervous system
CT	-	Computed tomography
CTNNB1	-	Catenin beta 1
CXCR3	-	Chemokine receptor 3
D/N	-	Desmoplastic / nodular
DAG	-	Diacylglycerol
DDR1/2	-	Disoidin domain receptors
ECIS	-	Electric cell-substrate impedance sensing
ECM	-	Extracellular matrix
EGF	-	Epidermal Growth Factor
EGFR	-	Epidermal Growth Factor Receptor
EGL	-	External granular layer
EMT	-	Epithelial-mesenchymal transition
Eph	-	Ephrin receptors
ERK 1 / 2	-	Extracellular signal-regulated protein kinase

ERM	-	Ezrin, radixin and moesin
ESC	-	Embryonic stem cells
EVL	-	Mena and Ena/VASP-like
FAK	-	Focal adhesion kinases
FFPE	-	Formalin fixed and paraffin embedded
FGF	-	Fibroblast Growth Factor
FGFRs	-	FGF receptors
FGFRL1	-	FGF like receptor 1
FN	-	Fibronectin
FOXH1	-	Factors like forkhead box H1
FoxO	-	Forkhead transcription factor
FRS2	-	Fibroblast receptor substrate 2
FSO	-	Federal Statistical Office
GAB1	-	GRB2 associated binding protein 1
GABA	-	*gamma*-Aminobutyric acid
GCKs	-	Germinal center-like kinases
GDFs	-	Growth differentiation factors
GDNF	-	Glial cell line derived neurotropic factor
GFAP	-	Glial Fibrillar Acidic Protein
GFP	-	Green fluorescent protein
GM-CSF	-	Granulocyte-monocyte colony stimulating factor
GNPCs	-	Granule Neuron Precursor cells
GnRH	-	Gonadotropin-releasing hormone
Grb2	-	Growth factor receptor-bound protein 2
GS	-	Gly / Ser regulatory
GTP	-	Guanosine triphosphate
G-TsF	-	Glioblastoma-derived T cell suppressor factor
HDAC	-	Histone deacetylase
HGF	-	Hepatocyte Growth Factor
HGK	-	Hepatocyte progenitor kinase-like kinase
HS	-	Heparin sulphate
HSP90	-	Heat shock protein 90
ICCC	-	International Classification of Childhood Cancer
iFGFs	-	Intracellular FGFs
IFN-α	-	Interferon-α
Ig	-	Immunoglobulin
IGF-1	-	Insulin-like Growth Factor-1
IGL	-	Internal granule layer
Ihh	-	Indian hedgehog
IL	-	Interleukin
IP3	-	Inositol triphosphate
I-SMADs	-	Inhibitory SMADs
JNK	-	c-Jun N-terminal kinase
KCNA1	-	Potassium voltage-gated channel subfamily A member 1
KLPH	-	αKlotho, βKlotho and Klotho-LPH related protein

LAP	-	Latency-associated proteins
LIMK	-	LIM kinase
LLCs	-	Large latent complexes
LMD	-	Leptomeningeal dissemination
LOX-1	-	Lectin-like oxidized-low-density lipoprotein receptor-1
LTBPs	-	Latent TGF-β binding proteins
mAb	-	Monoclonal antibody
MAP4K4	-	Mitogen Activated Kinase kinase kinase kinase 4
MAPK	-	Mitogen Activated Protein Kinase
MAT	-	Mesenchymal-amoeboid transition
MB	-	Medulloblastoma
MBEN	-	Medulloblastoma with extensive nodularity
MCP-1, MCP-3	-	Macrophage chemoattractant protein 1 and 3
Med PDX	-	Medulloblastoma patient-derived xenografts
MEKK1	-	Mitogen activated protein kinase kinase kinase 1
MKKs	-	MAP kinase kinases
MLCK	-	Myosin light-chain kinase
MMPs	-	Matrix metalloproteinases
MRCK	-	Myotonic dystrophy kinase-related Cdc42-binding kinases
MSCs	-	Mesenchymal stem cells
mTOR	-	Mammalian target of rapamycin
NCI	-	National cancer institute
NFκB	-	Nuclear factor kappa-light-chain-enhancer of activated B cells
NGF	-	Nerve growth factor
NICER	-	National Institute for Cancer Epidemiology and Registration
NIK	-	Nck interacting kinase
NLS	-	Nuclear localization sequence
NO	-	Nitric oxide
NSAIDS	-	Non-steroidal anti-inflammatory drugs
NT	-	Neurotrophic factor 4
PAK	-	p21-activated kinase
PDGF-B	-	Platelet-derived growth factor receptor B
PDX	-	Patient derived xengrafts
PFS	-	Posterior fossa syndrome
PGF	-	Polypeptide growth factors
PI3K	-	Phosphatidylinositol-4,5-bisphosphate 3-kinase
PKC	-	Protein kinase C
PLC-γ	-	Phosolipase C gamma
PlGF-1	-	Placental growth factor 1
PNET	-	Primitive neuo-ectodermal tumours
PPCs	-	Pericyte progenitor cells
PTB	-	Phosphotyrosine binding
PTCH1	-	Protein patched homolg 1
PtdIns	-	Phosphoinositides
PTK	-	Protein tyrosine kinase

PYK2	-	Proline rich tyrosine kinase 2
Rac1	-	Ras-related C3 botulinum toxin substrate 1
Raf	-	Rapidly Accelerated Fibrosarcoma
RAS	-	Rapidly activate p21
RhoA	-	Ras homolog gene family, member A
ROCK	-	Rho associated protein kinase
R-SMADs	-	Regulatory SMADS
RTK	-	Receptor tyrosine kinase
RTKi	-	Receptor tyrosine kinase inhibitors
SCCR	-	Swiss Childhood Cancer Registry
SDF-1	-	Stromal-cell derived factor
SEER	-	Surveillance, Epidemiology and End results
SH2	-	Src-homology 2
SH3	-	Src-homology 3 domain
Shh	-	Sonic hedgehog
SK6	-	Phospho-p70 S6 Kinase
SARA	-	SMAD Anchor for Receptor Activation
SMADs	-	SMA/MAD homology
SMO	-	Smoothened
SOS	-	Son of Sevenless
SPRY	-	Sprouty
NGFR	-	Nerve growth factor receptor
Src kinase	-	Proto-oncogene tyrosine-protein kinase Src
STAT3	-	Signal transducer and activator of transcription 3
STAT5	-	Signal transducer and activator of transcription 5
TAK1	-	TGF-β1 activated kinases
TEM	-	Tie-2 expressing monocytes
TGFR	-	Transforming growth factor receptor
TGF-β	-	Transforming Growth Factor-β
T_H17	-	IL-17 expressing pro-inflammatory T helper cells
TIEG-1	-	TGF-β inducible early gene-1
TLRs	-	Toll like receptor
TNF-α	-	Tumour necrosis factor-alpha
TRAF	-	6TNF receptor associated factor 6
TβRI	-	TGF-β receptor I
TβRII	-	TGF-β receptor II
TβRIII	-	TGF-β receptor III
uPA	-	Urokinase plasminogen activator
VASP	-	Vasodilator stimulated phosphoprotein
VEGF	-	Vascular Endothelial Growth Factor
VT	-	Valine and threonine
WASP	-	Wiskott-Aldrich syndrome protein
WHO	-	World Health Organization
YAP1	-	Yes associated protein 1

8. Acknowledgements

- Acknowledgements

8. Acknowledgements

I acknowledge my deep sense of gratitude to my supervisor PD Dr. Martin Baumgartner for giving me the opportunity to join his exciting laboratory to pursue my PhD work. I thank him for his unstinted efforts in giving his expertise and inputs wherever necessary and guiding me throughout this work. He had been very kind and patient while suggesting me the various possibilities of this project and correcting my doubts. I very much appreciate his overall moral support both during stressful and good times. I thank him for all the stimulating scientific discussions, which always motivated me to dream 'BIG' and achieve in the field of cancer research. I also thank him for giving me the freedom to follow my research ideas and for all the great solutions he provided for any upcoming scientific and non-scientific challenges.

I am equally grateful to Dr. Michael Grotzer for providing his valuable clinical expertise in the field of Medulloblastoma. I thank him for the infinite discussions during 'Wednesday Lab meetings' and for reading and correcting my manuscripts. He has never let me forget the 'Translational - Big picture' in cancer research and has constantly encouraged me to explore the various translational research aspects of my project.

I express my thanks to my committee members PD Dr. Jean-Pierre Bourquin, Prof. Dr. Lucas Pelkmans, Prof. Dr. Gisbert Schneider and Prof. Dr. Britta Engelhardt for their continuous guidance and helpful comments. The thesis committee meetings have been full of constructive discussions and critical advices, which aided in the proper design of experiments and successful completion of this project.

Thanks and appreciation to all the past and present members of the neuro-oncology team: Dr. Tarek Shalaby, Dr. Giulio Fiaschetti, Dr. Min Ma, Katja Egli, Carolin Kordomatis, Ramona Scherrer, Gustavo Alencastro Veiga Cruzerio, Elena Alvarez, Dr. Anuja Neve, Dimitra Tripolitsioti, Jessica Migliavacca and Charles Capdeville for their contributions to my project and for providing a creative ambience to do research. It has been a great honour to be a part of the neuro-oncology team and especially over the last years, it has been such a supportive team. I thank all my colleagues for the wonderful friendship we share apart from research and for all fun-filled moments throughout my PhD. A special thanks to Dimitra Tripolitsioti for patiently teaching me the biochemical techniques in the lab and for her moral support outside the lab.

My sincere thanks to the Cancer Biology PhD program, Life Science Zürich Graduate School and University of Zürich for its help and support to carry out this project. I acknowledge all my other colleagues in August-Forel strasse 1 and EICR department for all the help I received with regard to various aspects of my project. I truly appreciate the critical comments and discussion during EICR meetings, which helped in the betterment of my project and my presentation skills. I owe my gratitude to our collaborators at ETH Zürich and at SickKids, Toronto, without whom this project would have not been possible. I thank Dr. Max Pillong and Dr. Jens Kunze for their help in dealing with software used in my project.

I thank my parents, my lovely sister (Poornigha), my brothers (Pradeep Kumar, Ashok and Shiva), my best friend (Madhumithah) and my cousins (Kaviya and Monish) for their unceasing support and encouragement throughout this work.

My journey in field of cancer research has been an exciting 'roller-coaster' ride from India to UK and now to Switzerland. Since the beginning of this journey, it has always been my passion to work on cancer metastasis and my dream to make the lives of cancer patients better through my research. I am immensely glad that my PhD aided me to pursue my passion. I would like to express my deepest appreciation to all the people who inspired, encouraged and provided the confidence to carry forward my passion and achieve my dream. Thanks for making me believe in my dreams. I realized that it is worth to chase my dreams. After all, dreams do come true; mine just did!!!

9. Auxiliary

- List of credit points
- Teaching hours
- Curriculum vitae

University of Zurich[UZH] | ETH zürich

List of activities

Student	Karthiga Santhana Kumar
Matriculation number	
Faculty/Department	Faculty of Science (UZH) (PhD student)

Title# *Module number*	*Category/* *Type*	*Lecturer/* *Offered by*	*Location/* *End date*	*Credits/* *Hours*
Scientific Integrity	Compulsory activity Transferable skills	LSZGS	Zurich 2014-03-11	0.00 0 h
5th Cancer Biology PhD program student retreat	Elective activity Summer school / Retreat	Cancer Biology PhD program PhD program	Filzbach am Walensee, Switzerland 2014-02-14	1.00 25 h
6th CB PhD program student retreat	Compulsory activity Summer school / Retreat	Cancer Biology PhD program PhD program	Davos, Switzerland 2016-03-31	1.00 25 h
Organization of the 6th CB PhD program student retreat	Elective activity Summer school / Retreat	Cancer Biology PhD program PhD program	Davos, Switzerland 2016-03-31	1.00 25 h
6th Cancer Network Zurich retreat	Compulsory activity Summer school / Retreat	CB PhD program LSZGS	Emmetten, Switzerland 2015-04-14	1.00 25 h
Clinical Cancer Research	Compulsory activity Subject-specific matters	Cancer Biology PhD program PhD program	Zurich 2014-09-26	2.00 64 h
Competency Awareness	Elective activity Transferable skills	Dr. Monika Clausen LSZGS	Zurich 2016-11-25	1.00 40 h
Ethics in Neurosciences	Compulsory activity Subject-specific matters	Dr. Jackie Leach Scully UZH	Zurich 2013-05-24	1.00 25 h
Comprehensive course in flow cytometry	Core elective activity Subject-specific matters	Dr. Vinko Tosevski UZH	Zurich 2014-03-27	1.00 25 h
Post-Beginners German A1.2	Elective activity Other	lic.phil. Ursina Tones UZH	Zurich 2015-05-26	2.00 90 h
Beginners German A1.1	Elective activity Other	lic.phil. Anja Gredig UZH	Zurich 2014-12-18	2.00 90 h
ISPNO 2016	Core elective activity Conference	Society for pediatric neuro-oncology UZH	Liverpool, United Kingdom 2016-06-15	1.00 25 h
ISREC 2014	Core elective activity Conference	EPFL UZH	EPFL, Lausanne 2014-01-25	1.00 25 h
Practical course in advanced microscopy	Core elective activity Subject-specific matters	ZMB UZH and ScopeM ETH UZH	Zurich 2014-01-24	2.00 64 h
Organization of the 2nd CB PhD program mini symposium	Elective activity Summer school / Retreat	Cancer Biology PhD program PhD program	Emmetten, Switzerland 2015-04-13	1.00 25 h
Molecular and Cell Biology of Cancer	Compulsory activity Subject-specific matters	Cancer Biology PhD program PhD program	Zurich 2014-05-12	2.00 64 h
Project management for research	Elective activity Transferable skills	Dr. Pamela Alean-Krikpatrick LSZGS	Zurich 2016-09-27	1.00 25 h
Scientific writing	Compulsory activity Subject-specific matters	Cancer Biology PhD program PhD program	Zurich 2014-06-23	1.00 25 h
Voice training and presentation skills in the sciences and medicine	Elective activity Transferable skills	Dr. Janneke van Woerden LSZGS	Zurich 2016-10-21	1.00 25 h
Post-Beginners German A2.1	Elective activity Other	Language centre of ETH ETH	Zurich 2015-12-16	2.00 90 h
Post-Beginners German A2.2	Elective activity Other	Language centre of ETH ETH	Zurich 2016-06-01	2.00 90 h
Grant - Sassella foundation stiftung for cancer research	Elective activity	Sassella foundation	Zurich	1.00

University of Zurich[UZH] | ETH zürich

Title# Module number	Category/ Type	Lecturer/ Offered by	Location/ End date	Credits/ Hours
	Other	other	2015-12-21	25 h
Total				28.00 917 h

Signature of Official Supervisor: _____

Date: 16.1.2017

University of Zurich[UZH]

Division of Biology / Fachbereich Biologie

Summary of teaching activities

This form[1] is to be completed and signed by the PhD student at the **end of the doctorate**, endorsed by the responsible professor[2] (rP), and submitted to the Office of the Dean of Studies when registering for the doctoral examination.

Please send a copy to: University of Zurich, Academic Support Office ASO ("Studienkoordination des Fachbereichs Biologie"), Division of Biology Y13-J-01, Winterthurerstrasse 190, 8057 Zürich studienkoordination.biologie@uzh.ch

Name of PhD candidate:	Karthiga Santhana Kumar
Responsible professor	PD Dr. Martin Baumgartner
Start date of PhD	11 Mar 2013
End date of PhD	10 Mar 2017

Summary of teaching activities

Semester	Teaching activity in Module Number … (if applicable)	Hours
	Master Student	50
	BIO319 (Nov 2014)	25
	BIO319 (Nov 2015)	25
	TOTAL	100

Signatures

Date: 12. Jan. 2017 PhD Candidate K. Kall

Date 12. Jan. 2017 Responsible professor M. Baumgartner

(See page 2 for descriptions of which activities can and cannot count towards the teaching requirement)

[1] Download under www.biologie.uzh.ch/Studium/Studiengang/Doktorat.html

[2] The rP is an MNF faculty member or somebody with "Promotionsrecht" at the MNF. The rP normally heads the PhD committee.

University of Zurich[UZH]

Which activities count towards teaching hours?

- Actual time spent teaching students in laboratory practicals, classroom sessions, lectures, exercise classes, fieldwork etc.
- Preparation
- Correcting students' reports
- Supervising / coaching in semester projects, summer schools, research practicals
- Marking exams
- Overseeing exams
- Master's thesis supervision[3]
- Instructor in the "Life Science Zurich – Learning Center (LSLC)"[4]

Which activities do NOT count as teaching?

- Journal clubs
- PhD student seminars
- Modules primarily for PhD students and where they acquire credit points
- Introducing new PhD students to the laboratory equipment and procedures of the research group
- Progress reports

In general: all those activities in which PhD students in purely research institutes would be involved (such as, e.g., Max-Planck Institutes)

[3] The Division Council recommends counting between **0 and 50 hours per Master's thesis**. The responsible professor decides on the final allocation of hours depending on the extent of scientific benefit that the PhD student gains from the supervision of the Master student (the higher the benefit, the fewer the hours). Good supervision of a Master student incorporates important components of teaching and learning: **the allocation of 0 hours should not be practiced in such cases**.

It is not recommended that PhD students fulfill their teaching requirement exclusively with supervision of Master students.

[4] PhD students interested in this teaching opportunity should first consult the paper "Rules for the partial fulfillment of the MNF teaching requirement within the LSLC" in which they will find all the details

Karthiga SANTHANA KUMAR

Ph.D. Student
03 Dec 1989
Kreuzstrasse 17, 8008 Zurich, Switzerland
+41 78 646 69 69
Karthiga.kumar@kispi.uzh.ch
Skype: karthi.flora
Languages: Tamil (native), English (proficient), German (A2)

EDUCATION:

Mar 2013 – Present: **PhD Neuro-oncology, University of Zurich, Switzerland**

- **Project:** Targeting cell dissemination and motility of medulloblastoma cells
- **Course work:** Molecular basis of cancer, clinical cancer therapy, scientific writing, ethics in neuroscience

Sep 2011 – Sep 2012: **M.Sc Cancer immunology and Biotechnology, The University of Nottingham, UK**

- **Marks obtained:** 72% (1st class with distinction, Second in the department among 30 students)
- **Thesis:** High expression of connexin43 and intratumoral T cells are associated with better survival in colorectal cancer
- **Course work:** Tumour immunology, cancer vaccines, antibody engineering, big-pharma business plan and models

Sep 2007 – May 2011: **B.Tech Biotechnology, Anna University, India**

- **Marks obtained:** 87.29% (1st class with distinction)
- **Thesis:** Evaluation of Anti-oxidant effect of Cocoa and Green tea polyphenols in human erythrocytes – A comparative study
- **Course work:** Cancer biology, cell and molecular biology, genetic engineering, biopharmaceutical technology

Jun 2005 – May 2007: **Higher secondary education (Standard X to XII), Kalaimagal Viddyalaya matriculation higher secondary school, India**

- **Marks obtained:**
  - **Standard XII -** 88.5% (1st class with distinction, Second in school among 120 students)
  - **Standard XI -** 92.7% (1st class with distinction, First in school among 130 students)
  - **Standard X -** 89.2% (1st class with distinction, Fourth in school among 160 students)
- **Course work:** Mathematics, physics, chemistry, biology

HANDS-ON EXPERIENCE:

- **PhD:** 3D cell culture, 3D drug screening, High throughput 2D and 3D migration and invasion assays, live cell video microscopy, confocal microscopy, immunoprecipitation, G-LISA, q-RT-PCR, Lenti-CRISPR
- **Masters:** Western Blotting, FACS, Immunohistochemistry, ELISA and SPSS
- **Bachelors:** Gel electrophoresis, solvent extraction for polyphenols, various biochemical tests for free radical damage and lipid peroxidation quantification

GRANTS, SCHOLARSHIPS AND AWARDS:

- Sassella foundation grant for cancer research, 50K CHF
- Swiss cancer league funding for cancer biology student retreat, 5K CHF
- Travel Grant from Cancer Biology PhD program for ISPNO 2016, 1K CHF
- Recipient of Developing solution masters scholarship, 7K GBP
- Rashtrapati Award in Bharath Scouts and Guides (National level - Presidential Award)
- RajyaPuraskar Award in Bharath Scouts and Guides (State level - Governor Award)

CONFERENCES:

Jun 2016: ISPNO 2016, Liverpool, United Kingdom
Poster: aCDc finds bFGF-induced dissemination antagonized by TGF-beta in medulloblastoma cells

Feb 2014: ISREC2014: Metastatic Colonization EPFL symposium, Lausanne, Switzerland
Poster: Targeting cell motility and dissemination of medulloblastoma cells

PUBLIC COMMUNICATIONS:

Oral Presentations:

Jan 2017, Jan 2015: Swiss pediatric oncology group meeting, Lugano, Switzerland
Oct 2016, Oct 2015: Children's research centre retreat, Zürich, Switzerland
Apr 2016, Oct 2014: Colloquium of applied cancer research, Zürich, Switzerland
Jan 2016, Jan 2014: Cytomeet, Bern, Switzerland
Jun 2014: Joint cancer meeting, Cancer network Zürich, Switzerland

Poster Presentations:

Jun 2015: Swiss image based screening conference, Basel, Switzerland
Jan 2015: C.B. Brupbacher symposium, Zürich, Switzerland
Oct 2014: Children's research centre retreat, Zürich, Switzerland

CANCER BIOLOGY PHD PROGRAM AND CANCER NETWORK RETREATS:

Mar 2016: 6th Cancer biology PhD program student retreat, Davos, Switzerland
Talk: Automated cell dissemination counter: A novel tool for experimental and prognostic quantification of tumour cell dissemination

Apr 2015: 6th Cancer network Zurich retreat, Emmetten, Switzerland
Poster: 3D Cell based diagnostic tool identifies novel anti-metastatic therapy targets in medulloblastoma

Feb 2014: 5th Cancer biology PhD program student retreat, Filzbach am Walensee, Switzerland
Poster: Targeting cell motility and dissemination of medulloblastoma cells

TEACHING EXPERIENCE:

Aug 2015 - Jan 2016: Supervisor - Medical PhD thesis, Medical student, University of Zürich
Nov 2015, Nov 2014: Course assistant - Cancer cell motility (BIO319), University of Zürich

SCIENTIFIC PUBLICATIONS:

- **Kumar, K. S.** *et al.* **Computer-assisted quantification of motile and invasive capabilities of cancer cells**. *Sci. Rep.* **5**, 15338; DOI: 10.1038/srep15338 (2015)
- **Karthiga Santhana Kumar** *et al*. **The Ser/Thr kinase MAP4K4 drives c-Met-induced motility and invasiveness in a cell-based model of SHH medulloblastoma.** SpringerPlus (2015) 4:19 DOI 10.1186/s40064-015-078

SPECIALISED COURSES AND IN-PLANT TRAININGS:

Courses:

- Practical course in advanced microscopy at ETH Zurich, Switzerland
- Comprehensive course on flow cytometry at University of Zürich, Switzerland
- Project management for researchers, Graduate school, University of Zürich, Switzerland
- Voice training for scientific presentations, Graduate school, University of Zürich, Switzerland
- Competency awareness, Graduate school, University of Zürich, Switzerland

In-plant trainings:

- Preliminary Tests and Microbiology at Dr. Kamakshi Memorial Hospital Pvt. Ltd, India
- Biological Databases and Tools at Helix info systems, India
- Basic and Advanced rDNA Techniques at Biozone Research Technologies, India

INTERPERSONAL SKILLS:

Possess excellent capability to organise events and to work in a team

- Have been a part of the organizing team and have organized the cancer biology PhD program student retreat (2016) Davos, Switzerland
- Organized the cancer biology PhD program mini symposium (2015) Emmetten, Switzerland
- Actively organized the national level biotechnology symposium (2010), Chennai, India

Effective in writing newsletter/magazine articles. Possess exceptional editing and designing skills

- Member - editorial board of the cancer network Zurich newsletter -"SCOOPED" since 2015
- Editor of the department of biotechnology magazine - "JET HELIX" from 2008 to 2011
- Member - editorial committee of the annual magazine of my school from 2000 to 2007

Leadership qualities and team building

- Have been a member of Bharath Scouts and Guides movement from 2000 to 2007 in which I have been elected as a team leader consecutively from 2004 to 2007 whereby I led my team of 8 members in various camps and interschool competitions

SOFTWARE SKILLS:

Adobe InDesign, Adobe After effects, Adobe Photoshop, CoralDraw, Graphpad Prism, ImageJ

NON-ACADEMIC INTERESTS:

Aerobics, Indian classical dancing, photography, drawing & painting, travelling